5. PHARMACOLOGICAL SCREENING OF

GISEKIA PHARNACEOIDES

5.1 ACUTE ORAL TOXICITY STUDY

5.1.1 Introduction

Medicinal plants play a key role in the human health care because of their efficacy, safety and lesser adverse effects. When herbal drugs are used as a therapeutic substance for treating various ailments, it becomes an essential requirement to fulfill the guidelines formulated by World Health Organization (WHO). WHO guidelines have given one of the important criteria to establish the safety profile of the herbal preparation (WHO report, 1991),

Numerous plants have been screened for their various biological activities in an attempt to replace the standard drugs. However, the herbal preparations have shown to control different diseases (Argueta et al., 1994) such as diabetes (Virdis et al., 2003), cancer (Hecker, 1968), syphilis, asthma, chest infections, rheumatism (Correia, 1994), leprosy (Bunkill 1985); hypertension (Tchikaya et al., 2003), mouth ulcer and throat problems, gastrointestinal disorders (kudi et al., 1999; Akinpelu 2001 Goncalves 2005; Taylor 2005) etc. Most of these plant species have not been subjected to chemical, toxicological, pharmacological or clinical investigation and have been ignored by national health authorities for many decades. The lack of
standardization of ingredients and their preparation usually are limited by the toxicity or relative lack of efficacy compared with standard medications.

Hence in the present study the petroleum ether, chloroform and methanolic extracts of Gisekia pharnaceoides whole plant obtained in succession were subjected to acute oral toxicity evaluation in order to assess, whether the extracts have any adverse effects or toxic manifestations for long term use. Toxicological study results play an important safety assessment for pharmaceuticals, pesticides and other chemicals. This study therefore was designed to evaluate their toxicity in Wister albino rats as toxicity may result from the overdose of these extracts.

5.1.2 Materials and methods

Preparation of the plant extracts for experimental studies

The crude extracts of Gisekia pharnaceoides in petroleum ether, chloroform and methanol are designated as GPP, GPC and GPM respectively as mentioned in the scheme I in Chapter 3. These extracts were used in the form of suspension in water with 1% sodium carboxy methyl cellulose (SCMC) as the suspending agent, for oral administration in rats.

Animals

Wister albino mice of either sex (25 – 30 g) and Wister albino rat of either sex (180 – 200 g) were obtained from the inbred colony of Department of pharmacology, C.L.B.M College of pharmacy, Thorapakkam, Chennai-96. The animals were kept in polypropylene cages at 25±2°C with relative
humidity of 45 – 55% under 12 h light and 12 h dark cycles. They were fed with standard laboratory animal fed obtained from (Poultry research station, Tamil Nadu Veterinary and Animal Science University, Chennai, India) and tap water ad libitum.

All the pharmacological and toxicological experimental protocols were approved by the Institutional Animal Ethics Committee (IACE) on Committee for the Purpose of Control and Supervision on Experimentation on Animals (CPCSEA), vide sanction letter No IAEC 21 / XV / CLBMCP.

The procedure was followed by using OECD (Organization of Economic Cooperation and Development) guidelines 423 (Acute toxic class method). Preliminary acute toxicity tests did not show any toxic signs or symptoms in rats administered up to 2000 mg Giseka pharnaceoides extract per kg body weight. In this assay, 4 groups consisting of control, methanolic extract, petroleum ether extract, chloroform extract-treated with 6 animals in each group each containing an equal number both male and female were formed. A dose limit at 2000 mg/kg body weight of each extract dissolved in sodium carboxy methyl cellulose (SCMC) was administered orally to the animals of test group. The animals of control group received SCMC. In each case the volume administered was 10 ml / kg body weight. Following the administration, the animals were closely observed during the first 3 h, and 48 h, there after, 14 days, for toxic sign and symptoms such as convulsions, salivation, diarrhea, lethargy, sleep and coma and death. The weight of the animals was monitored during the experimental period.
5.1.3 Results

No abnormality in the general behaviour of the test animals either in the short term or long term, was observed. Treated animals exhibited normal behaviour as that of control group. No death was observed in any of the group and all the animals lived upto 14 days (Table. 8). There was no body weight loss during the observation period. But all the animals exhibited a gain in body weight (Table. 9). The daily consumption of food and water in each sex and group was observed to exhibit the same pattern.

5.1.4 Discussion

In general, folk medicine uses plant extracts without taking into account their toxicity aspects. Though the *Gisekia pharnaceoides* is reported to be used as an anthelmintic agent in folklore medicine (Wealth of India 2002), the toxicity of this plant have not been studied so far. In the present study, the observations of acute toxicity test revealed that all the three extracts of this investigation upto 2000mg/kg body weight showed no toxicity. According to Clarke and Clarke (1977), substances with LD$_{50}$ of 1000 mg/kg body weight per oral route are registered as being safe or low toxicity. Therefore, the *Gisekia pharnaceoides* which did not register LD$_{50}$ even upto 2000m/kg body weight in the present investigation may be regarded as non toxic either in folk medicine or in the diet. The result of Tables 8 and 9 also suggest that the solvent extracts of *Gisekia pharnaceoides* did not affect the increase in body weight as well as water and food intake, indicative of non-toxic nature of the plant.
5.2 ANTI-INFLAMMATORY ACTIVITY

5.2.1 Introduction

Inflammation or phlogosis is a pathological response of mammalian tissues to a variety of hostile agents including infectious organisms, toxic chemical substances, physical injury or tumor growth leading to local accumulation of plasmic fluid and blood cells (Sobota et al., 2000). Although inflammation is a defense mechanism, the complex events and mediators involved in the inflammatory reaction can induce maintain and aggravate many disorders. Hence, the employment of anti-inflammatory agents may be helpful in the therapeutic treatment of those pathologies associated with inflammatory reactions (Sosa et al., 2002). The clinical treatment of inflammatory diseases is dependent on drugs which belong either to the non-steroidal or steroidal chemical therapeutics. The non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, indomethacin and ibuprofen inhibit early steps in the biosynthesis pathway of prostaglandins by inhibition of COX enzymes and are the main drugs used to reduce the untoward consequences of inflammation (Albert et al., 2002). However, the side effects of the currently available anti-inflammatory drugs pose a major problem in their clinical use. For instance, NSAIDs cause several serious adverse effects like gastric injury and ulceration, renal damage and bronchospasm due to their non-selective inhibition of both isoforms of the COX enzyme (Tapiero et al., 2002). The use of steroidal drugs as anti-inflammatory agents is also
becoming highly controversial due to their multiple side effects (Van den Worm et al., 2001). Therefore, a need arises for the development of newer anti inflammatory agents from natural sources with more powerful activity and with lesser side effects as substitutes for chemical therapeutics.

Data concerning the inflammatory disease in the rural area is sometimes, scarce. It is therefore not surprising that inflammatory diseases are the common health problems treated with traditional remedies which mainly comprise medicinal plants (Degif and Hahn 2001). For instance several plants species of *seseli* growing in China are used in folklore for treating the inflammatory diseases like swelling rheumatism, pain and common cold (Hu et al., 1990). *Sphenocentrum jollyanum*, herbal species of *menispermeaceae* family has been reported to be of use for the treatment of rheumatic pains in Nigeria. (Moody et al., 2006). *Cinnamomum camphora sieb*, commonly known as camphor as long been prescribed in traditional medicine for the treatment of inflammation related diseases such as rheumatism, sprains, bronchitis, asthuma and muscle pains. The materia medica and wealth of India indicates the names of several plants having anti-inflammatory property. In this direction the present study contributes more on therapeutic use of plants for treating inflammatory disorders. The plant identified was *Gisekia pharnaceoides* belong to the family of *molluginaceae*. The aim of this part of the work is to test the various extracts of *Gisekia pharnaceoides*, viz. GPP, GPC and GPM extract for the anti-inflammatory activities *in vivo*.
**Principle**

The inflammatory reaction is readily produced in rats in the form of paw edema with the help of irritants or inflammagens. Substances such as carrageenan, formalin, bradykinin, histamine, 5-hydroxy tryptamine, mustard and egg white, when injected in the dorsom of the foot in rats produce acute paw oedema within a few minutes of the injection.

5.2.2 **Material and Methods**

GPP, GPC and GPM were separately made into suspension SCMC. A 1% suspension of diclofenac sodium in SCMC was prepared, as reference drug.

**Carrageenan induced hind paw edema model**

**Dose fixation**

A pilot study was performed in which anti inflammatory activity by carrageenan induced paw oedema in rat model. Wistar albino rats of either sex were divided into six groups (n=6). The extracts were administered orally in geometric progression starting from 100 mg/kg, and 250 mg/kg 500 mg/kg, 750 mg/kg and 1000 mg/kg respectively. The volumes of edema of the injected and contra lateral paws were measured at +1, 2, 3 and 4 h after induction of inflammation using a plethysmometer and percentage of anti-inflammatory activity was calculated.
Anti-inflammatory activity of test was performed at two dose levels. In case of the petroleum ether, chloroform and methanolic extracts of *Gisekia pharnaceoides*, the dose selected was 250 mg/kg, and 500 mg/kg based upon the dose fixation studies. The reference drug diclofenac sodium, the dose is fixed as 5 mg/kg.

**Treatment protocol**

The Wistar albino rats of either sex were divided into eight groups containing six animals in each group and were fed with SCMC (Control) or the crude extracts in SCMC (Test) or the diclofenac sodium in SCMC (Reference) orally, as mentioned below. The test group of rats received either 250 mg or 500 mg of crude extract per kg body weight.

Group I - 1% SCMC 10 ml/kg/ body weight - Control
Group II – GPP 250 mg/kg body weight
Group III -GPP 500 mg/kg/ body weight

Group IV - GPC 250 mg/kg/ body weight - Test
Group V - GPC 500 mg/kg/ body weight

Group VI - GPM 250 mg/kg/ body weight
Group VII - GPM 500 mg/kg/ body weight

Group VIII- Diclofenac sodium 5 mg/kg body weight - Reference

The carrageen an assay procedure was carried out according to the method of Winter *et al.*, 1962. Oedema was induced by injecting 0.1 ml of a 1% solution of carrageenan in saline into the plantar aponeurosis of the left
hind paw of the rats. The extracts or reference drug or the control vehicle was administered 60 min prior to the injection of the carrageenan. The volumes of edema of the injected and contra lateral paws were measured at +1, 2, 3 and 4 hrs after induction of inflammation using a plethysmometer. Results were expressed as percentage inhibition oedema, calculated according to the following formula: (Garcia et al., 1995)

\[
\text{Percentage inhibition} = \frac{\text{Control-Test}}{\text{Control}} \times 100
\]

5.2.3 Results

In the carrageenan induced rat hind paw oedema test, the average left back paw volume and percentage of inhibition by different extracts and the reference drug are given in Table.10. For the Control (untreated) group the injection of carrageenan caused localized oedema well in advance compare to other groups. In this untreated group, the swelling increased progressively to a maximum volume at 3rd h after the carrageenan injection. The rats pre-treated with the crude extracts of *Gisekia pharnaceoides* had significant reduction in the oedema volume on 3rd h post dosing, at all dose levels used. The percentage inhibition was as high as 73% for 500 mg/kg body weight GPM of *Gisekia pharnaceoides* at 3 h. The effect of GPM administration in controlling the paw oedema was almost comparable to the reference drug (Table 10 Fig 25). An increase in the volume of paw oedema was observed upto 3 h in all the groups, and there after it decreased. GPM at a
dose level of 500 mg/kg body weight showed highest percentage inhibition of (73.68%) oedema followed by the GPP that exhibited 63.15%. The GPC seemed to be less effective than the other two extracts. The highest percentage inhibition for 250, 500 mg/kg body weight dose was observed to be 56.5 and 73.68 % respectively.

5.2.4 Discussion

The anti inflammatory effect of the different solvent extracts of *Gisekia pharnaceoides* (GPP, GPC and GPM) were investigated in the present study. The carrageenan test was selected because of its sensitivity in detecting orally active anti-inflammatory agents particularly in the acute phase of inflammation (DiRosa *et al.*, 1971; DiRosa, 1972). The intraplantar injection of carrageenan in rats leads to paw oedema reportedly in two different phases: the initial phase which occurs between 0-2 h after injection of carrageenan has been attributed to the release of histamine, serotonin and bradykinin on vascular permeability (Vinegar 1987). Where as the late phase has been reported to be complement dependent reaction due to the over production of prostaglandin in tissues (DiRosa *et al.*, 1971). Also increased production of nitric oxide (NO) and prostaglandins E₂ (PGE₂) has been noted in carrageenan challenged animals (Selvemini *et al.*, 1996). Though the *Gisekia pharnaceoides* is known as a dietary supplement among the rural folk, its pharmacological effects have not been explored yet. The data presented in this report indicate that *Gisekia pharnaceoides* can exert a significant immuno modulatory effect on various inflammatory responses, though the exact mechanism is yet to be
understood. According to the results, the different extracts of *Gisekia pharnaceoides* were shown to possess a different degree of anti-inflammatory activity which is again dose dependent.

The variation of anti-inflammatory activity among the crude extracts of *Gisekia pharnaceoides* may be due to the compositional variation of the individual extract. For instance, the anti-inflammatory activity of coumarins in TPA-induced ear oedema model in mice depends on their individual substitution on the aromatic ring (Garcia-Argaez et al., 2000). The observed anti-inflammatory property of the extracts of Gisekia *pharnaceoides* might possibly due to their ability to inhibit the reactive oxygen species. As the kaempferol (a member of flavonoid family), free radical scavenger (Panichayupakaranant and Kaewsuwan 2004), this property would in turn result in the inhibition of lipid peroxidation and subsequent inflammation, that is noticed in the form of swelling.

Accumulating evidence indicates that excessive production of NO plays a pathogenic role in both acute and chronic inflammation (Clancy 1998). NO is responsible for the vasodilatation, increase in vascular permeability oedema formation and including synthesis of prostaglandins at the site of inflammation (Moncada1991; Grisham 1999). The role of NO in relation to carrageenan induced paw oedema was also reported (Selvemini 1996). Manipulation of NO free radical can be a potential and promising therapeutic area in treating inflammations. Approaches being currently used for inflammatory disorders include NO scavenger as well as NO inhibition
Since all the test drugs (GPP, GPC and GPM) exhibited significant \textit{in-vitro} NO free radical scavenging activity as discussed in the section 5.4.3. The same may be, in part, attributed to the observed anti-inflammatory effect of these test drugs. The increased rate of inhibition of oedema Fig. 26 displayed GPM its constituent is actively involved in the reactions of first phase as well as the second phase of the inflammatory response. The inhibition in the first 2 h (ie first phase) was observed to be 53\% and from 2-3 h (second phase) the inhibition increased further to 73\% showing the active participation of GPM in the anti-inflammatory reactions. Where as the response recorded by the extracts of GPP and GPC are much slower compared to that of GPM (Fig.25) and more particularly in the second phase (after 2h). However, the pretreatment by GPP and GPC seemed to have strengthened the defence mechanism and there by the paw oedema was reduced to as high as 58\% in the initial stage itself, and there after dose not seem to have participated in the anti-inflammatory response.

5.3 ANALGESIC ACTIVITY

5.3.1 Introduction

The analgesics are one of the highest therapeutic categories on which research efforts are concentrated (Elisabetsky and Castillhos 1990) in recent years. Analgesic compounds available in the market, still present in wide range of undesired effects (Katzung, 2001) leaving an open door for new and better compounds. Natural products are believed to be an important source of new chemical substance with potential therapeutic applicability. A large
number of Indian medicinal plants are attributed with various pharmacological activities due to their diversified phytochemical content. Most of these medicinal plants have been identified and described by different authors (Chopra et al., 1956). In this direction, attempt has been made in this study to identify an undisclosed plant with analgesic activity. The efficacy of the crude extract of the plant of this study is reported here.

**Principle**

Exposing heat to mice tail is one way of stimulating pain in cutaneous receptors, which in turn excites the thermo selective and nociceptive fibers (Luttinger 1985). Immersion of rat tail in hot water provokes an abrupt withdrawal of the tail from the heat source. The time difference in the withdrawal of tail of opioid and other rest drug treated animal was monitored. Tail immersion method is most widely and reliably used for revealing the potency of opioid analgesics (Jansen 1963).

**Animals**

Swiss albino mice of either sex were used for the experiments study.

5.3.2 **Materials and Methods**

GPP, GPC and GPM were separately made into suspension of SCMC. Morphine prepared in the form suspension in SCMC of 1% (w/v) was administered orally to animals.
**Dose fixation**

In order to fix the dose for the extracts of *Gisekia pharnaceoides*, a pilot study was performed in which analgesic activity by tail immersion method was chosen as the model. Albino mice of either sex were divided into six groups (n=6). The extracts were administered orally in geometric progression at a dose of starting from 50 mg/kg, 100 mg/kg, and 250 mg/kg, 500 mg/kg, 750 mg/kg and 1000 mg/kg body weight respectively. The basal reaction time was recorded and the % production of analgesia was calculated.

Analgesic activity of test was performed at two dose levels. In case of GPP, GPC and GPM extracts of *Gisekia pharnaceoides*, the dose selected was 250 mg/kg, and 500 mg/kg based upon the dose fixation studies. The reference drug morphine, the dose is fixed as 5 mg/kg.

**Tail immersion method**

Tail immersion test was conducted as described by Aydin *et al* (1999). Eight groups of Swiss albino mice of either sex weighing between 20-25 gm were used for the study. This involved immersing the extreme 3cm of the mice tail in water bath containing water at 55 ± 0.5°C followed by the observation for tail flicking of mice. The basal reaction time was monitored by observing tail flicking. The interval of time between the dipping and flicking of tail is noted as basal reaction time. The test groups were given GPP, GPC or GPM SCMC 250, 500 mg/kg p.o in SCMC or SCMC alone.
(Control). The reference group animals received morphine (5 mg) in SCMC. In a different expression, the animals were divided and treated as follows.

Group I - 1% SCMC 10 ml/kg body weight - Control
Group II – GPP 250 mg/kg body weight
Group III – GPP 500 mg/kg body weight
Group IV – GPC 250 mg/kg/ body weight - Test
Group V – GPC 500 mg/kg body weight
Group VI – GPM 250 mg/kg body weight
Group VII – GPM 500 mg/kg body weight
Group VIII - Morphine 5mg/kg body weight - Reference

After administration of the drugs, the tail flick response was tested at an interval of 30, 60, 90,120,150 and 180 minutes. A cut off period of 10 sec is considered as a maximum analgesic and the tail is removed from source of hot water to avoid the damage.

5.3.3 Results

A significant reduction of the painful sensation due to tail immersion in warm water was observed following oral administration of the different solvent extracts at a dose of 250 and 500 mg/kg. The effect was seemed to be noticeable after a period of 30 min (Fig. 27) and it was dose dependent (Table. 11). The heat tolerance of the animal was observed to be low in all the three solvent extract (GPP, GPC and GPM), with respect to 250 mg/kg dose compare to that of 500 mg/kg dose (Fig. 28). However, the basal reaction
time recorded by these low dose fed animals is higher than that of the control (SCMC) group. (Fig. 28 and Table.11). The inhibitory effect of the extracts became more pronounced between 60-120 min post dosing. Of all the experimental samples, the methanol extract have shown highest analgesic activity compared to other extracts for a particular dosage, in the tail immersion test.

5.3.4 Discussion

Compare to the crude extracts of Gisekia pharnaceoides, morphine a centrally active analgesic drug (Heidary and Darban 1999; Hajhashemi et al., 2002), produced increased analgesic effect in the tail immersion method. Therefore, the potency of the crude extracts (250 or 500 mg) was lower than morphine (5 mg). However, this composition cannot be made as the matched quantity of the active principle (Kaemphferol) of Gisekia pharnaceoides was not administered to the mice in this study. The calculations, based on the yield, say that each gram of crude extract contains 2 mg kaemphferol the biologically active substance. In other words, 1 mg of biologically active substance is present in 500 mg crude extract. Therefore it is clear that test animals were administered with 0.5 mg or 1 mg of active substance as against 5 mg morphine in the control group was able to pronounce a basal reaction time of 5.83 sec for 500 mg extract (Table. 11) as against 6.83 sec for morphine (Fig. 28). The phytochemical studies (Chapter 3) Gisekia pharnaceoides revealed the presence of many chemicals such as alkaloids, phenolic compounds, flavonoids and tannins in the crude extracts. Determination of the
role of each compound in the analgesic effect of this plant is a wide field for more investigation. But the presences of flavanoids in *Echium amoenum* have been reported to possess analgesic activity *in vivo* (Delorme *et al.*, 1977; Milis and Bone 2000). The presence of flavanoids in an extractable amount in the *Gisekia pharmaceoides* was detected and therefore the analgesic effect observed in this study may be attributed to the flavanoids, particularly the kaempferol. This finding is in good agreement with that of the earlier investigators who have worked on other plant species. (Pathak *et al.*, 1991; Meyre- Silva *et al.*, 1999; Bittar *et al.*, 2000). These authors claim that the flavanoids may have action on the peripheral anti-nociceptive effect. The kaempferol being the major component of the crude extract of *Gisekia pharmaceoides*, this flavanoid could be responsible for the peripheral anti-nociceptive effect. However, the exact mechanism of action of flavanoids remains to be explored.

5.4 **IN-VITRO ANTIOXIDANT ACTIVITY**

5.4.1 Introduction

*Gisekia pharmaceoides*, the wild plant is already a part of the diet of the rural population in certain parts of India. However, the pharmacological activity of this plant is yet to be reported. Many plants often contain substantial amounts of antioxidants including vitamins C and E, carotenoids, flavonoids and tannins etc. and thus can be utilized to scavenge the excess free radicals from human body (Pratt 1992; Shahidi *et al.*, 1992; Rice–Evans *et al.*, 1996). Many investigations indicate that these compounds are of great
value in preventing the onset and/or progression of many human diseases (Halliwell and Gutteridge 1989; Halliwell et al., 1992; Willet 1994; Tsao and Akthar 2005). The oxidative stress in a biological system is exerted by the free radicals like superoxide, hydroxyl, nitric oxide etc., which are known a Reactive Oxygen Species (ROS). Most living species have a number of defence mechanism against oxidative stress and toxic effects of normal oxygen metabolism. In the case of plants, when used for edible purpose, it is necessary to determine the efficacy of natural antioxidants to combat the free radicals that are involved in the development of degenerative diseases (Campbell and Abdulla 1995). Thus total antioxidant capacity of an edible vegetable could be a parameter to evaluate the quality of vegetable foods (Arnao et al., 1998; Cano et al., 1998), apart from its nutritional value. Hence, this study was designed for the evaluation of possible beneficial antioxidant potency of Gisekia pharnaceoides extracts by employing different methods and techniques. The free radical system employed here are DPPH, Nitric oxide, hydroxyl, superoxide and ABTS.

5.4.2 Materials and Methods

The crude extracts of Gisekia pharnaceoides in petroleum ether, chloroform and methanol are designated as GPP, GPC and GPM respectively as mentioned in the scheme I in Chapter 3.
5.4.2.1 Assay of diphenyl picryl hydrate radical scavenging

Principle

1,1, Diphenyl 1,2-picryl hydrazyl (DPPH) is a stable free radical which shows deep violet colour in ethanol solution, characterized by an absorption maximum, at 517 nm. When a solution of DPPH is mixed with that of a substance that can donate a proton, the free radical DPPH is reduced to hydrazine.

![Chemical structure of DPPH and hydrazine conversion](image)

Basically this technique is a decolourization assay which evaluates the absorbance decrease at 517 nm, produced by the addition of the antioxidant to a DPPH solution in ethanol. DPPH assay is considered as a valid and easy assay to evaluate scavenging activity of antioxidants, since the radical compound is stable and does not have to be generated as in other type of radical systems.

Method

This assay is based on the measurement of the scavenging ability of antioxidant substances towards the stable radical. In this experiment the free
radical scavenging activity of the crude extracts was examined \textit{in-vitro} by using DPPH radical (Yokazawa \textit{et al.}, 1998). Extracts were prepared with different concentration from a maximum of 800 to minimum of 25 μg/ml. Briefly, the reaction mixture consisted of 1 ml of 0.1 mM DPPH in ethanol, 0.95 ml of 0.05 M Tris-HCl buffer (pH 7.4), 1 ml of ethanol and 0.05 ml of the extract. The absorbance of the mixture was measured at 517 nm exactly 30 sec after adding the crude extracts. The experiment was performed, in triplicate, and % scavenging was calculated using the formula:

\[
\text{Percentage inhibition} = \frac{\text{Control-Test}}{\text{Control}} \times 100
\]

Parallely, a blank was made with all the reagents except the extracts. The activity was compared with Vitamin E, which was used as a reference antioxidant.

\textbf{5.4.2.2 Assay of nitric oxide scavenging activity}

Nitric oxide (NO) is a hydrophobic molecule and highly diffusible free radical, generated through the oxidation of L-arginine to L-citrulline by nitric oxide synthase (Alderton \textit{et al.}, 2001). Low concentration of nitric oxide acts as a signaling molecule with dichotomous regulatory roles in many physiological process, whereas high level of nitric oxide produced by inflammatory cells can damage DNA, RNA, lipids and proteins, leading to increased mutations and altered enzyme and protein function important to multi stage carcinogenesis process (Patel \textit{et al.}, 1999, Li and Wogan, 2005).
Effects of nitric oxide on cell is ultimately dependent on a complex of factors including existing biological milieu; rate of nitric oxide production and its rate of diffusion; interaction with other free radicals, metal ions and proteins; levels of protective enzyme such as catalase and superoxide dismutase; levels of antioxidants such as glutathione. However, the fate of nitric oxide in biological systems is broadly governed by three main reaction processes: diffusion and intracellular consumption, auto oxidation to form nitrous anhydride (N₂O₃) and the reaction with superoxide to form peroxynitrite (ONOO⁻) (Chen et al., 2001).

Principle

The assay is based on the principle that, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions and the same can be estimated using Griess Ilosviy reaction (Garrat et al., 1964). In the present investigation, Griess Ilosviy reagent is modified by using 0.1% (w/v) naphthylethylene diamine (NED) dichloride, instead of 5% (w/v) 1-naphthylamine scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. (Marcocci et al., 1994).

Method

Sodium nitroprusside (5μM) in standard phosphate buffer solution was incubated with different concentration (25-800 μg/ml) of the extracts dissolved in standard phosphate buffer (0.025 M, pH 7.4) and the tubes were
incubated at 25°C for 5 h (Sreejayan and Rao, 1997). After 5 h, 0.5 ml of incubated solution was removed and diluted with 0.5 ml Griess reagent (prepared by mixing equal volume of 1% sulphanalamide in 2 % phosphoric acid and 0.1% naphthal ethylene diamine dihydrochloride in water). The absorbance of chromophore formed during diazotization of nitrite with sulphanalamide and its subsequent coupling with naphthyl ethylene diamine was read at 546 nm. The control experiment was also carried out in similar manner, using distilled water in the place of extracts. The experiment was performed in triplicate and % scavenging was calculated using the formula:

$$\text{Percentage inhibition} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100$$

Parallely, a blank was made with all the reagents except the extracts. The activity was compared with Vitamin E, which was used as a reference antioxidant.

5.4.2.3 Measurement of Hydroxyl radical scavenging activity

Principle

Hydroxyl radical (OH’) scavenging activity can often be calculated using the “deoxyribose assay”: a mixture of ferric chloride (FeCl₃) and ethylenediamine tetra acetic acid (EDTA) in the presence of ascorbate reacts to form iron (II)-EDTA and oxidized ascorbate, H₂O₂ then reacts with iron (II)-EDTA to generate iron (III)-EDTA plus HO’ in the so-called Fenton reaction.
Fe$^{2+}$ + H$_2$O$_2$ $\rightarrow$ Fe$^{3+}$ + OH$^-$ + HO$^*$

That radical not scavenged by other components of the reaction mixture attack the sugar deoxyribose and degrades it into a series of fragments, some or all of which react on heating with thiobarbituric acid at low pH to give a pink chromogen. Thus the scavenging activity towards OH$^*$ of a substance added to the reaction mixture is measured on the basis of the inhibition of the degradation of deoxyribose (Halliwell 1990; Aruoma et al., 1993).

**Method**

The hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and the extracts for hydroxyl radicals generated in the Fe$^{3+}$/ascorbate/EDTA/H$_2$O$_2$ system. The hydroxyl radicals attack deoxyribose, which eventually results in TBARS formation (Elizabeth et al., 1990). The reaction mixture contained deoxyribose (2.8 mM), FeCl$_3$ (0.1 mM), EDTA (0.1 mM), H$_2$O$_2$ (1 mM), ascorbate (0.1 mM), KH$_2$PO$_4$-KOH buffer (20 mM, pH 7.4) and various concentration (25-800 μg/ml) of extracts and reference drug (Vitamin E) in a final volume of 1 ml. The reaction mixture was incubated for 1 h at 37°C. Deoxyribose degradation was measured as TBARS (Thiobarbituric acid reactive substance) at 532 nm (Ohkawa 1979) and percentage scavenging was calculated using the formula:

\[
\text{Percentage inhibition} = \left(1 - \frac{\text{Control-Test}}{\text{Control}}\right) \times 100
\]
Parallely, a blank was made with all the reagents except the extracts. The activity was compared with Vitamin E, which was used as a reference antioxidant.

5.4.2.4 2, 2-Azinobis-(3-ethylbenzothiazoline - 6 - sulphonic acid) (ABTS) radical cation decolorization assay

Principle

The ABTS assay is based on the inhibition by antioxidants of the absorbance of the radical cation ABTS$. The ABTS$ radical cation is generated directly by chemical reduction by manganese dioxide in the absence of hemeprotein and H$_2$O$_2$ (Rice-Evans and Miller 1997a). A modification of this method has been introduced by a decolorization technique in which the radical is generated directly in a stable form using potassium / ammonium persulphate (Re et al., 1999). Afterwards the formed radical is mixed with antioxidant in the reaction medium and the percentage inhibition at 745 nm is calculated.

Method

The antioxidant capacity of each extract was evaluated by studying its ability to bleach the radical (ABTS$). ABTS radical cation was produced by reaction of ABTS solution (7 mM) with 2.45 mM, ammonium persulfate and the mixture was allowed to stand in a dark room temperature for 12 -16 h before use. In this study, different concentrations (25-800 µg/ml) of the crude extract (0.5 ml) were added to 0.3ml of ABTS solution and the final volume
was made up with ethanol to make 1 ml. The absorbance was read at 745 nm (Auddy et al., 2003; Miller 1997) and the percentage scavenging calculated by using the formula as given below:

\[
\text{Percentage inhibition} = \frac{\text{Control-Test}}{\text{Control}} \times 100
\]

Parallely, a blank was made with all the agents except the extracts. The activity was compared with Vitamin E, which was used as a reference antioxidant.

5.4.2.5 Assay of superoxide radical scavenging activity

Principle

The scavenging activity towards superoxide radical of a wide range of antioxidants is measured in terms of inhibition of generation of \( \text{O}_2^- \) with the hypoxanthine-xanthine oxidase superoxide generating system (Robak and Gryglewski, 1988; Halliwell, 1990; Mitsuya et al., 1990). In this method \( \text{O}_2^- \) is generated using a non-enzymatic reaction of potassium superoxide in the presence of alkaline DMSO (Nishikimi et al., 1972; Robak and Gryglewski, 1988). This \( \text{O}_2^- \) reduces nitro-blue tertazolium (NBT) into formazan at pH 7.4 and room temperature, and the formazan generation is followed by spectrophotometry at 560 nm. Any added molecule capable of reacting with \( \text{O}_2^- \), inhibits the production of formazan.
Method

The method was performed by using alkaline Dimethyl sulphoxide (Henry et al., 1976). Potassium superoxide and dry DMSO were allowed to stand in contact for 24 h and the solution was filtered immediately before use. A 200 µl of the filtrate was added to 2.8 ml 10 mM potassium phosphate buffer containing 56 µM nitroblue tetrazolium (NBT) and 10µM EDTA. To this, crude extract of Gisekia pharnaceoides (1 ml) of various concentrations (25 – 800 µg/ml) was added and the absorbance was recorded at 560 nm, against a control in which pure DMSO was added instead of alkaline DMSO and the percentage scavenging calculated by using the formula as given below:

\[
\text{Percentage inhibition} = \frac{\text{Control-Test}}{\text{Control}} \times 100
\]

Parallely, a blank was made with all the agents except the extracts. The activity was compared with Vitamin E, which was used as a reference antioxidant.

5.4.3 Results

Several concentrations, ranging from 25 – 800 µg/ml, of the methanolic, chloroform and petroleum ether extracts of Gisekia pharnaceoides were tested for their antioxidant activity in different in vitro models. It was observed that free radicals were scavenged by the test
compounds in a concentration dependent manner up to 800 µg/ml concentration in all the models (Table 12 – 16 and Fig 29 - 33). In GPM the maximum percentage inhibition in all the models viz, ABTS, DPPH, hydroxyl radicals, nitric oxide and super oxide radical was found to be 87.79, 77.78, 65.45, 63.62 and 51.78% respectively at 800 µg/ ml. In GPC the percentage inhibition in all these models was found to be 60.83, 58.24, 52.13, 49.14 and 45.49 % respectively at 800 µg/ ml. In GPP percentage inhibition in all the models was found to be 55.66, 50.54, 44.86, 42.08 and 40.42% respectively at 800µg/ml. On comparative basis the methanolic extract showed better activity in quenching ABTS radicals with an IC$_{50}$ value of 150 µg/ml and DPPH radicals with an IC$_{50}$ 155 µg/ml. However, the extracts show encouraging response in quenching hydroxyl radicals with an IC$_{50}$ value of 150, 180 and 250 µg/ml respectively for GPM, GPC and GPP. The quenching activity was moderate against the remaining oxidant models investigated in this study.

5.4.4 Discussion

Oxidative stress has been implicated in the pathology of many diseases and conditions including diabetes, cardiovascular disease, inflammatory conditions, cancer and aging (Marx $et al.$, 1987). Antioxidants may offer resistance against oxidative stress by scavenging the free radicals, inhibiting the lipid peroxidation and by many other mechanisms and thus prevent diseases (Youdim and Joseph 2003).

DPPH is a relatively stable free radical. The assay is based on the measurement of the scavenging ability of antioxidants towards the stable
radical DPPH. From the present results it may be postulated that *Gisekia pharnaceoides* reduces the radical to the corresponding hydrazine when it reacts with the hydrogen donors in the antioxidant principles. Such observations have also been made by Sanchez-Moreno (2002) in his studies on the free radical scavenging activity in foods and biological systems. From methodological point of view, the DPPH method is recommended as easy and accurate with regard to measuring the antioxidant activity of fruit and vegetable juices or extracts. The results are highly reproducible (Gil 2000; Cano 1998). The results presented in Table 12 and Fig 29. suggest that the solvent extracts of *Gisekia pharnaceoides* must be a source of reducing equivalents, and therefore donates an electron to reduce the DPPH radicals to its corresponding hydrazine (Bolis *et al*., 1958).

Nitric oxide is a free radical produced in mammalian cells, involved in the regulation of various physiological processes (Lalenti 1993 & Ross 1993). However, excess production of NO is associated with several diseases such as cancer (Nguyen 1992), diabetes (Corbett 1992), trauma (Szaboc 1994), sepsis, (Thiemermann 1997) atherosclerosis, multiple sclerosis (Parkinson 1997) and arthritis and in inflammations (Ohshima 2003). Herbal drugs having nitric oxide radical scavenging property are gaining importance (Sarkar 2005; Rao 2006) because they possess various biological activities related to antioxidant mechanisms (Shirwaikar *et al*., 2004). In the present study the nitrite produced by the incubation of sodium nitro pruside in standard phosphate buffer at 25°C was reduced by the extracts of *Gisekia pharnaceoides* (Table 13 and Fig 30). This may be due to the antioxidant principles in the extracts which compete
with oxygen to react with nitric oxide (Marcocci et al., 1994) thereby inhibiting the generation of more deleterious products such as nitrous anhydride (N₂O₃) and perhydroxy nitrite (ONOO⁻) (Chen et al., 2001).

Ferrous salts can react with hydrogen peroxide and form hydroxyl radical via Fenton’s reaction. The iron required for this reaction is obtained either from the pool of iron or the heme-containing-proteins (Cotran 1999)). The hydroxyl radical (OH⁻) thus produced may attack the sugar of DNA deoxy causing ribose fragmentation; base loss and DNA strand breakage (Kaneko 1999). The generation of OH⁻ in the Fenton reaction is due to the presence of iron ions. When the Fe²⁺ / Fe³⁺ redox couple is bound by certain chelators, the OH⁻ formation is prevented. Whereas the increased colour formation in the absence of crude extracts were observed in the deoxyribose assay, as has been observed by (Sanchez-Morero 2002). Hence it is presented that the crude extracts of this investigation acts as the chelators of iron ions, binding to them, and preventing the formation of radical, though the extracts not directly involved in the OH⁻ scavenging. The results (Table 14 and Fig 31) indicate that the extracts of *Gisekia pharnaceoides* play a major role in the inhibition of ribose fragmentation and hence the decreased colour formation in the deoxyribose assay.

The ABTS assay is applicable for both lipophilic and hydrophilic antioxidants. The assay is based on the inhibition of the absorbance of the radical cation, ABTS⁺⁺, which has a characteristic long wavelength absorption spectrum (Sanchez-Morero 2002 ; Miller and Rice- Evans 1997). The ABTS
chemistry involves direct generation of ABTS radical mono cation with no involvement of any intermediary radical. It is a decolorization assay, thus the radical cation is performed prior to addition of antioxidant test system, rather than the generation of the radical taking place continually in the presence of antioxidants. The results (Table 15 and Fig 32) obtained implied the activity of the extract either by inhibiting the formation of or scavenging the ABTS•+ radicals since both the inhibiting and scavenging properties of antioxidants towards ABTS•+ radicals, have been reported (Re 1999; Rice-Evans 1997) earlier. The results of this study were compared with that of the vitamin E which is used as a reference scavenger.

Super oxides are produced from molecular oxygen by the oxidative enzymes (Sainani et al., 1997) of body as well as via non enzymatic reaction such as auto oxidation by catecholamines (Hammani et al., 1998). Superoxide dismutase catalyses the dismutation of the highly reactive superoxide anion to oxygen and hydrogen peroxide (Kamalakkannan et al., 2003). Superoxide anion is the first reduction product of oxygen (Ray et al., 2002) which is measured in terms of inhibition of generation of $O_2^-$. The various phyto compounds of Gisekia pharmaceoides, that are observed in the preliminary phytochemical screening, including tannins (Lin et al., 1974; Yoshida et al., 1989), polyphenols (Brand Williams et al., 1995; Bondet, et al., 1997; Yokozawa et al., 1998) and flavanoids (Okawa et al., 2001; Cao et al., 1997a) are reported to have antioxidant properties and hence, these compounds may be responsible for the scavenging of superoxide, generated potassium superoxide-alkaline DMSO system, in addition to other reactive species.
studied in this investigation. The overall superoxide radical scavenging activity of the various extracts viz GPM, GPC and GPP suggest (Table 16 and Fig 33) that the GPP exhibit increased activity compared to other two extracts probably due to its higher tannin content, as tannins are easily extractable in low polar solvents, such as petroleum ether, compare to high polar solvents like chloroform and methanol.

The free radical scavenging property of the crude extracts of *Gisekia pharnaceoides* against DPPH, NO, OH⁻, O₂⁻ and ABTS⁺ radicals is clearly understood from the results of this chapter. The phytochemical screening of the extract (Chapter 3) revealed the presence of flavonoids as well as tannins and other compounds. Further purification of the crude extract ended up with one compound namely kaempferol, confirmed the presence of flavonoids in *Gisekia pharnaceoides*. Therefore the antioxidant property exhibited by the crude extract against the radicals studied might be due to the tannins and flavonoids. Such observation has been made by earlier investigators for methanolic extract of other plants (Ho *et al.*, 1992; Wang *et al.*, 1996). Since the free radical scavenging properties of the extracts have been confirmed *in vitro*, the *Gisekia pharnaceoides* as such, by all means should also be potential antioxidant *in vivo*. In human body superoxide, nitric oxide and hydroxyl free radicals are produced endogenously. These radicals of normal metabolism cause extensive damage to DNA, proteins and lipids and constituting a major contribution to aging and also to degenerative diseases of aging such as cancer, cardiovascular disease, brain dysfunction, and cataracts (Ames *et al.*, 1993). This oxidation process could be prevented or delayed, if the antioxidants or the anti oxidant rich food is added to the diet. *Gisekia*
in this study, is found to be an effective free radical scavenger and hence can serve as a potential antioxidant diet for those in the advancement of age or aging related disorders and diseases.

Flavanoids are natural products, which has been shown to posses various biological properties related to antioxidant mechanisms (Perrisoud 1982; Glyglewski 1987; Corvazier 1985; Middleton 1984; Robak 1988 and Ratty 1998). Thus, the antioxidant potential of *Gisekia pharnaceoides* observed is due to the presence of flavonoids therein.

5.5 WOUND HEALING ACTIVITY

5.5.1 Introduction

**Wound:** Wound is defined as a loss or breaking of cellular and anatomic or functional continuity of living tissues (Ayello, 2005). Experimental wounds can be categorized into two types namely, full and partial thickness. While the partial thickness wounds heal by mere epithelialization, the healing of full thickness wound which extends through the entire dermis involves more complex well regulated events (Treget *et al.*, 1997; Taun *et al.*, 1998). Skin injury is highly complex owing to its structural complexity. The skin is the most affected organ following an injury. Skin consists of usually a cellular epidermis and acellular connective tissue dermis and these two layers are interconnected through the basement membrane or basal lamina. Skin, may therefore, be considered as an interesting structure in which wound healing involves both regeneration and repair, and the most common site of injury (Melissa Calvin 1998).
Wound healing: Cutaneous wound healing is a self motivated physiological process of cell regeneration which occurs without any external stimuli. The process of wound healing occurs in different phases such as hemostasis, inflammation, proliferation and tissue remodeling (Fig 34). Each phase overlaps with the next (Manley and Bellmann 2000).

1. Homeostasis

Tissue injury initiates a response that first clears the wound of devitalized tissue and foreign material, setting the stage for subsequent tissue healing and regeneration. The initial vascular response involved a brief and transient period of vasoconstriction is followed by active vasodilation accompanied by an increase in capillary permeability. Platelets aggregated with in a fibrin clot secrete a variety of growth factors and cytokines that set the stage for an orderly series of events leading to tissue repair.

2. Inflammation

The Inflammation phase, present itself as erythema, swelling and warmth and is often associated with pain. The inflammatory response increases vascular permeability, resulting in migration of neutrophils and monocytes into the surrounding tissue. The neutrophils engulf debris and microorganisms, providing the first line of defense against infection. Neutrophils ceases after the first few days of post injury if the wound is not contaminated. If this acute inflammatory phase persists, due to wound hypoxia, infection, nutritional deficiencies, medication use, or other factors
related to the patients immune response, it could interfere with the late inflammatory phase (Stadelmann et al., 1998). In the late inflammatory phase, monocytes are converted in the tissue to macrophages, which digest and kill bacterial pathogens, scavenge tissue debris and destroy remaining neutrophils. Macrophages, begin the transition from wound inflammation to wound repair by secreting a variety of chemotactic and growth factors that stimulate cell migration, proliferation and formation of the tissue matrix.

3. Proliferation

Proliferative phase is dominated by the formation of granulation tissue and epithelialization. Its duration is dependent on the size of the wound. Chemotactic and growth factors released from platelets and macrophages stimulate the migration and activation of wound fibroblasts that produce a variety of substances that is essential for wound repair, including glycosaminoglycans (mainly hyaluronic acid, chondroitin-4 sulphate, dermatan sulphate, heparin sulphate) and collagen (Stadelmann et al., 1998). This forms an amorphous, gel like connective tissue matrix necessary for cell migration. New capillary growth must accompany the advancing fibroblasts into the wound to provide metabolic needs. Collagen synthesis and cross–linkage is responsible for vascular integrity and strength of new capillary beds. Improper cross-linkage of collagen fibers has been responsible for non-specific post operative bleeding in the patients with normal coagulation parameters. Early in proliferation phase, fibroblast activity is limited to cellular replication and migration. Around the third day after wounding, the
growing masses of fibroblast cells begin to synthesize and secrete measurable amount of collagen. Collagen levels rise continually for approximately three weeks. The amount of collagen secreted during this period determines the tensile strength of wound.

4. Wound tissue remodeling

The final phase of wound healing is wound remodeling; the events in remodeling are responsible for the increase in tensile strength, decrease in erythema and scar tissue bulk, reorganization of new collagen fibres and the final appearance of the healed scar. This process continues up to two years, achieving 40 – 70 percent of the strength of undamaged tissue at four weeks (Stadelmann et al., 1998). Type I collagen becomes the major collagen present in the mature scar or normal skin, reversing the earlier type III collagen predominance.

Factors involved in wound healing

In most of the cases, the complication in wound healing is due to inflammation. Inflammation results in a continuous generation of reactive species, such as the super oxide radical or the non radical hydrogen peroxide (Pawlak et al., 1998). An imbalance between oxygen species and the antioxidant defence mechanisms of a cell, leading to an excessive production of oxygen metabolites leads to condition of oxidative stress. Oxidative stress results in lipid peroxidation, DNA breakage and enzyme inactivation including free radical scavenging enzyme (Wisemann et al., 1996). Evidence
for the potential role of antioxidants in the pathogenesis of many diseases suggests that antioxidants may be of therapeutic use in these conditions (Skaper et al., 1997). Flavanoids, a group of naturally occurring benzo-γ-pyrone derivatives, have been shown to posses several biological properties, including hepatoprotective, antithrombotic, anti inflammatory and antiviral activities, many of which may be related partially at least to their antioxidants and free radical scavenging ability (Chen et al., 1990).

Fig. 34. The phases of cutaneous wound healing
5.5.2 Materials and Methods

(i) The *Gisekia pharnaceoides* extracts of methanol (GPM), chloroform (GPC) and petroleum ether (GPP) were prepared as mentioned in Chapter 2.

(ii) Soframycin ointment was purchased locally.

(iii) Simple ointment containing as placebo wool fat (5%) hot paraffin (5%), cetosteryl alcohol (5%) and white soft paraffin (85%) was prepared (Anonymous, 1953) and used as placebo control.

(iv) Preparation of test ointment:- About 50 gm of semisolid plant extract was incorporated into the 1000 g of simple ointment base.

5.5.2.1 Wound creation and treatment

Albino rats weighing 150-180 g were used for the study. Rats were maintained in individual metabolic cages throughout the experiment, in hygienic conditions and they were fed with commercial balanced diet and water ad libitum. The fur on the backside of the rats was shaved under mild ether anesthesia. Subsequently, open excision wounds of standard size of 2 x 2 cm were made by using a template. The ointment was applied daily twice a day till the wound completely healed (Chatterjee and Chakravorty, 1993). The progress of wound healing was evaluated periodically by monitoring the histological changes of the skin tissue obtained at 5th, 10th and
15th days after wound creation. The anti-oxidant level after complete healing of the wound was also assayed.

The animals were divided into five groups containing six animals each and were treated as given below.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Groups</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Group I</td>
<td>Control - simple ointment</td>
</tr>
<tr>
<td>2</td>
<td>Group II</td>
<td>GPM extract ointment</td>
</tr>
<tr>
<td>3</td>
<td>Group III</td>
<td>GPC extract ointment</td>
</tr>
<tr>
<td>4</td>
<td>Group IV</td>
<td>GPP extract ointment</td>
</tr>
<tr>
<td>5</td>
<td>Group V</td>
<td>Soframycin ointment</td>
</tr>
</tbody>
</table>

5.5.2.2 Wound contraction assessment by planimetry

The contour of the individual wounds of both control and experimental animals was periodically measured using transparent graph sheet and the rate of healing was calculated and expressed as percentage contraction (Morgen 1994). The observation on percent wound contraction and epithelialization period were measured from initial day (Rashed et al., 2003). The following formula was used to calculate the percentage of wound contraction.

\[
\text{Wound contraction} \% = \left( \frac{\text{Wound area on day 1} - \text{wound area on day 'n'} }{\text{Wound area on day 'n'} } \right) \times 100
\]
5.5.2.3 Histological studies

Skin tissue from the wounded site of individual rat was removed after sacrifice. They were then fixed in 10% buffered formalin, dehydrated through graded alcohol series (30 - 100%), cleared in xylene and embedded in paraffin wax (m.p. 56°C). Serial sections of 5 µm thickness were cut using microtome, and stained with hematoxylin – eosin and were examined under light microscope for epithelialization, fibrosis and angiogenesis (Yeo et al., 2000).

5.5.2.4 Preparation of skin homogenate

A 10% homogenate of skin tissue was prepared in 0.02M Tris HCl buffer pH 7.0 using a Teflon homogenizer in ice-cold condition. The homogenate was centrifuged at 5000 rpm for 10 min. The supernatants collected were used for the determination of non-enzymatic antioxidants such as ascorbic acid (AA) and enzymatic antioxidants such as catalase (CAT) and superoxide dismutase (SOD) and glutathione peroxidase (GPx). Apart from this, malondialdehyde (MDA), the product of lipid peroxidation was also determined using the supernatant.

5.5.2.5 Protein determination

Protein content of the skin homogenate was determined according to the method of Lowry et al, (1951). The aromatic amino acids tyrosine and tryptophan present in proteins react with Folin –Ciocalteau reagent, which contains phosphomolybdtic acid and phosphotungstate to produce a blue colour compound, which shows absorption maximum at 660 nm.
Reagents

Lowry’s reagent

Solution A : 2% sodium carbonate in 0.1N NaOH
Solution B : 0.5% copper sulphate in 1% sodium potassium tartarate
Solution C : 50 ml of solution A was mixed with 1.0 ml of solution B, just before use
Folin–Ciocalteau reagent: 1:2 dilutions with water just before use
Standard solution : Standard bovine serum albumin (BSA) containing 20 mg /100 ml were prepared.

Procedure

A 0.1 ml of the sample was made upto 1.0 ml with water, and a 4.5 ml of lowry’s reagent (Solution C) was added, mixed well and allowed stand for 10 min. To this, 0.5 ml of Folin–ciocalteau reagent was added, mixed well and kept at room temperature for 20 min. A standard solution containing BSA at a concentration of 20 – 100 μg and reagents blank were treated in a similar manner. The colour developed was read at 660 nm.

The total protein was expressed as mg/100mg of tissue.
5. 5.2.6 Measurement of antioxidant level

5. 5.2.6.1 Estimation of activity of Glutathione peroxidase

The activity of Glutathione peroxidase was estimated according to the method of Rotruck et al., (1973). Reduced glutathione (GSH) is converted into oxidized glutathione (GSSG) in the presence of the enzyme Glutathione peroxidase (GPx) and its reaction is as follows

$$2\text{GSH} + \text{H}_2\text{O}_2 \xrightarrow{\text{GPx}} \text{GSSG} + 2\text{H}_2\text{O}$$

Reagents

1. Phosphate buffer 0.1M, pH 7.0: 420 ml of 0.1M disodium hydrogen phosphate was mixed with 0.1M sodium dihydrogen orthophosphate until the pH adjusted to 7.0.

2. Ethylene diamine tetra acetic acid (EDTA), 1mM: 29.2 mg of EDTA was dissolved and made upto 100 ml using distilled water.

3. Sodium azide 10 mM: 65 mg of sodium azide was dissolved in distilled water and made upto 100 ml.

4. Glutathione (GSH), 4 mM: 125 mg of GSH was dissolved in 100 ml of distilled water.

5. Hydrogen peroxide, 2.5 mM: 0.03 ml of hydrogen peroxide solution (30%) was made up to 100 ml with water.
6. Trichloro acetic acid (TCA), 10%: 10 gm TCA was dissolved and made upto 100 ml with distilled water.

7. Disodium hydrogen phosphate (0.3 M): 4.25 gm of disodium hydrogen phosphate was dissolved and made upto 100 ml with distilled water.

8. 5, 5 – Dithio bis (2-nitrobenzoic acid) substrate (DTNB), 0.6mM: 2.37 mg of DTNB was dissolved in 100 ml of 1% tri sodium citrate.

9. Glutathione standard (200 µg/ml): 20mg of reduced glutathione was dissolved in 100ml of distilled water.

10. Trisodium citrate (1%w/v): 1gm of trisodium citrate was dissolved and made upto 100 ml with distilled water.

**Procedure**

Triplicate samples of, 0.1 ml of skin homogenate taken in separate tubes was mixed with 0.4 ml of phosphate buffer and added 0.1ml each of sodium azide, EDTA and H₂O₂. Then, 0.2ml of GSH was added to all the tubes and the reaction was arrested by the addition of 10 % TCA after 3 min incubation at 37°C intervals. The tubes were then centrifuged and 1.0 ml of the supernatant was transferred to fresh tubes. The blank contained 1ml of distilled water. The standard glutathione was prepared in separate tubes at a concentration range of 5 to 20 µg in a final volume of 1 ml. To all the above tubes 4 ml of disodium hydrogen phosphate solution was added followed by
0.5 ml of DTNB and the colour developed was read at 412 nm in a spectrophotometer against the blank.

The activity of Glutathione peroxidase is expressed as Units/mg protein. One unit of enzyme activity is defined as the amount of the enzyme that converts 1 µmole of GSH to GSSG per minute in the presence of H₂O₂.

5. 5.2.6.2  Ascorbic acid estimation

Ascorbic acid was estimated according to the method of Omeye et al., (1979). Ascorbic acid is oxidized by copper to form dehydro ascorbic acid and diketogluconic acid. These products are then treated with dinitrophenylhydrazine (DNPH) to form a derivative i.e., bis-2-4-dinitrophenylhydrazone. This compound, in strong sulphuric acid undergoes a rearrangement to form a yellowish orange product, which was measured spectrophotometrically.

Reagents

1. Trichloro acetic acid (TCA-10%): 10 g TCA was dissolved and made upto 100 ml with distilled water.

2. Dinitrophenylhydrazine / Thiourea / Copper sulphate (DTC) reagent: 0.4 g of thiourea, 0.05 gm of copper sulphate and 3.0 gm of DNPH was mixed and made upto 100 ml with 9N H₂SO₄.

3. Sulphuric acid H₂SO₄ (65%): 65ml of Conc.H₂SO₄ was made upto 100 ml with distilled water.
4. Sulphuric acid $\text{H}_2\text{SO}_4$ (9N): 25 ml of Conc.$\text{H}_2\text{SO}_4$ was made upto 100 ml with distilled water.

5. Ascorbic acid standard: A standard solution of ascorbic acid was made by dissolving 100 mg in 100 ml of distilled water.

**Procedure**

1.0 ml of skin homogenate was made upto 3.0 ml by addition of 10% TCA and the precipitated protein was cleared off by centrifugation. To 1.0 ml of the supernatant was added 0.5 ml of DTC reagent and was incubated at 37°C for 3 h. After incubation, 2.5 ml of ice cold 65% $\text{H}_2\text{SO}_4$ was added, mixed well and allowed to stand at room temperature for an additional period of 30min. The yellowish orange colour formed was measured at 520 nm. The standard tubes containing ascorbic acid were also treated in the same manner. Ascorbic acid values were expressed as mg/gm protein.

5.5.2.6.3 **Superoxide dismutase**

The activity of superoxide dismutase was estimated according to the method of Marklund and Marklund (1974). Pyrogallol, auto oxidizes rapidly in aqueous solution at a faster rate at a higher pH (8.0) to produce several intermediate products. The inhibition of pyrogallol auto oxidation by the enzyme present in the sample is employed in the quantification of activity of superoxide dismutase. The inhibition of auto oxidation brought about by the addition of enzyme is evaluated at the early stage as an increase in absorbance at 420 nm.
Reagents

1. Tris - HCl buffer (0.1M), pH 8.2: 1.576 g of Tris-HCl was dissolved in distilled water and the pH was adjusted to 8.2 using 1N NaOH and the volume was made up to 100 ml with distilled water.

2. Pyrogallol solution (0.2 mM): 126 mg of pyrogallol was dissolved in 100 ml of Tris- HCl buffer and stored in an aluminum foil wrapped stoppered test tube.

3. Diethylenetriamine penta acetate (DTPA-1mM): 19.67 mg of DTPA was dissolved in 100 ml of distilled water.

4. Ethylene diamine tetra acetic acid (EDTA-1mM): 29.22 mg of EDTA was dissolved in 100 ml of distilled water.

Procedure

A mixture containing 2.6 ml of Tris-HCl buffer, 0.1 ml of EDTA and 0.5 ml of DTPA was prepared. To this mixture, 0.5 ml of pyrogallol was added and the increase in absorbance was read at 420nm against the blank for 3 min to determine the rate of auto oxidation of pyrogallol. The test sample, 0.1 ml of skin homogenate taken in separate tube was mixed with 2.5 ml of Tris-HCl buffer, 0.1 ml of EDTA and 0.5 ml of DTPA. To this mixture, 0.5 ml of pyrogallol was added and the increase in absorbance was read at 420 nm using a spectrophotometer against the blank for a period of 3 min.
This measurement constituted the rate of inhibition of auto oxidation of pyrogallol brought about by the enzyme present in the tissue homogenate. The reagent blank contained a mixture of 3.1 ml of Tris-HCl buffer, 0.1 ml of EDTA and 0.5 ml of DTPA and this was used to set 100% absorbance.

The enzyme activity of superoxide dismutase was expressed as units/mg of protein and it is defined as the 50% inhibition of auto oxidation of pyrogallol per min by the enzyme.

5. 5.2.6.4 Estimation of activity of Catalase

The activity of catalase was estimated by the method of Sinha (1972). The method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of hydrogen peroxide (H₂O₂), resulting in the formation of perchromic acid as an unstable intermediate. The chromic acetate, thus produced was measured spectrophotometrically.

Reagents

1. Dichromate-acetic acid reagent (1:3 v/v): one volume of 5% potassium dichromate in water was mixed with 3 volume of acetic acid. The solution was further diluted to 1:5 with distilled water.

2. Phosphate buffer (0.01M, pH 7.0): 84 ml of 0.01M disodium hydrogen phosphate was mixed with 0.01M potassium dihydrogen orthophosphate until the pH adjusted to 7.0.
3. Hydrogen peroxide (0.2M): 0.02 ml of H$_2$O$_2$ (S.G-1.01) was taken and immediately mixed in 1 litre of distilled water. This solution was used for initiation of reaction with tissue homogenate.

4. Potassium dichromate, 5% (K$_2$Cr$_2$O$_7$, 7H$_2$O): 5 gm of K$_2$Cr$_2$O$_7$ was dissolved in distilled water and made upto 100 ml.

Procedure

Triplicate sample of 0.1 ml of skin homogenate was taken, to which 1.0 ml of phosphate buffer and 0.5 ml of hydrogen peroxide were added. The reaction was arrested immediately by the addition of 2.0 ml dichromate-acetic acid reagent at 0, 30, and 60 second intervals. The reagent blank was prepared by the addition of 1.6 ml of buffer and 2.0 ml of dichromate acetic acid reagent taken in separate tubes. The test and blank tubes were heated in boiling water bath for 10min for the green colour develop. The intensity of green colour developed was measured at 570 nm using spectrophotometer against blank, after the tubes were cooled at room temperature.

The activity of Catalase in the tissue homogenates is expressed as µmoles of H$_2$O$_2$ consumed / min/mg protein at 37°C.

5. 5.2.7 Estimation of Tissue lipid peroxidation

Lipid peroxidation products (as Malondialdehyde) were determined by the thiobarbituric acid reaction as described by Droper and Hadley (1990). Malondialdehyde, a secondary product of lipid peroxidation reacts with
thiobarbituric acid to form a pink chromogen (Thiobarbituric acid – 2 Malondialdehyde adduct), which was measured spectrophotometrically.

**Reagents**

1. **20% TCA solution**: 20 gm of TCA was dissolved in 100ml of double distilled water.

2. **0.12M thiobarbituric acid**: This was prepared freshly before use.

3. **Standard solution**: 1,1,3,3-tetramethoxy propane 100 nmoles/ml

**Procedure**

To 0.5 ml skin homogenate, 2.0 ml of 20% TCA was added. The contents were mixed well and centrifuged at 4000 rpm for 20 minutes. 2.0 ml of the supernatant was mixed with 2.0 ml of TBA reagent. Reagent blank and standards (5 – 20 nmoles) were also treated similarly. The contents were heated for 20 minutes in a boiling water bath the tubes were cooled to room temperature and the absorbance was read at 532 nm in a shimadzu UV-visible double beam spectrometer. The lipid peroxide content was expressed as nmoles MDA / mg protein.
5.5.3 Results

Rate of wound contraction

The percent wound contraction was calculated from the wound size, measured periodically on day 5, 10, 15. It was observed to be 91, 96 and 100% on day 15 for GPP, GPC and GPM treated groups respectively. The planimetry of the wound of the animals at different period of intervals was depicted in Table 17 & Fig 35. The results of this study show a remarkable difference between the control and GPM treated group. The other two extract treated groups viz., GPP, GPC also seem to have positive influence on the wound contraction but, are less effective compare to the GPM. The differences in the healing efficacy among the extracts are clearly seen in the first 10 days, after that the rate of healing is more or less same in all the three groups. The healing pattern of soframycin (Reference) treated group follows the results of GPM treatment. However, the real advantage of using an herbal drug can be assessed by the histological examination of the re-epithelialised skin specimens.

Period of epithelialization

The progression of wound contraction was evaluated by macroscopic observations and parallely, the contour of wound size was monitored by planimetry at regular intervals of time. The rate of wound healing in terms of wound closure was observed to be in the following order

Group II > Group V > Group III > Group IV > Group I
The macroscopic analysis of wound revealed that the methanolic extract (GPM) treated groups required a total period of 14 days for complete epithelialization, whereas the petroleum ether extract (GPP) treated group and chloroform extracted (GPC) treated groups required 18 and 16 days respectively for complete epithelialization.

**Histology**

Histological studies provided a good evidence of suitability of progression of wound healing. The status of epithelialization in the tissue was studied by examining hematoxylin-eosin stained specimen. The specimens were observed for re-epithelialization, migration of cells, distribution of collagen fibers, formation of blood vessels etc and a comparison was made among the treated and control groups, and are presented as below.

**Group I:** Figure 36 shows the histology of control specimen on 5\textsuperscript{th}, 10\textsuperscript{th} and 15\textsuperscript{th} days. The untreated wound specimen of this group shows that the process of healing is still in the early stage of inflammation (Fig 36.a) with few numbers of fibroblasts and absence of keratinization. Appearance of loosely packed fiber, incomplete epithelialization and presence of active fibroblasts are seen on 10\textsuperscript{th} day specimen of control. On 15\textsuperscript{th} day the specimen showed few numbers of blood vessels as a mark of angiogenesis in the dermis region.

**Group II:** Figure 37 show the histology of GPM treated specimen on 5\textsuperscript{th}, 10\textsuperscript{th} and 15\textsuperscript{th} days. The post wounding tissue on 5\textsuperscript{th} day (Fig 37.a) showed
undifferentiated keratinization with active fibroblast. The 10th day specimen showed (Fig 37.b) almost complete epithelialization with fairly good amount of collagen and neo-vascularization. Where as on 15th day there was an (Fig 37.c) uniform accumulation of collagen with prominent thick bundles consisting of moderate number of fibroblast. The epidermal layer appeared continuous, consisting of well defined epithelium and no inflammatory cells were observed in the wound site. In this group, the healing process was completed within 15 days.

**Group III:** Figure shows the histology of the tissue specimen of GPC treated group on 5th 10th and 15th days respectively. The specimens of day 5 (Fig 38.a) post wounding showed the onset of undifferentiated keratinization and few number of fibroblast. On day 10 was nearing to completion and few numbers of blood vessels are seen in the midst of and loosely packed collagen fibers in dermis region (Fig 38.b). Almost complete epithelialization, clearly differentiated epidermis, and closely packed but more irregular arrangement of collagen fibers are seen on 15th day showed (Fig 38.c).

**Group IV:** shows the histology of GPP treated wound tissue specimen on 5th 10th and 15th days respectively. The histological result of this group on day 5 (Fig 39.a) resembles the same as that of the Group III (Fig 38.a), revealing that it is in the process of inflammation. On day 10, the specimen showed the progression of the process of epithelialization, angiogenesis and collagen deposition (Fig 39.b). On 15th day (Fig 39.c) epithelialization was almost complete but with irregular and loose distribution of collagen in the
dermis region.

*Group V:* The histology of reference group (soframycin treated) specimens is shown in figures 40 a, 40.b and 40.c 50, 51 and 52 for 5th, 10th and 15th days respectively. The onset of keratinocyte differentiation and accumulated active fibroblasts are seen on 5th day (Fig 40.a). Specimen of 10th day (Fig 40.b) showed the progression of epithelialization towards completion with irregular deposition of collagenous substance and under-developed blood vessels. Complete epithelialization and uniform distribution with more active fibroblasts were seen in dermis region on day 15. The collagen deposition was still in the amorphous state (Fig 40.c) unlike that is seen in Group II (Fig 37.c).

**Protein content**

Table 18 shows the water soluble protein levels in the re-epithelialised tissue of treated and control animals. As seen from the results (Table 18), the Group II animals show higher level of protein compare to other groups. More particularly, it (Group II) shows significant increase against the protein value of control group (p<0.001).

**Antioxidant level**

**Glutathione peroxidase (GPx)**

Table 19 shows the glutathione peroxidase level in the skin tissue of different experimental groups. As shown in the results (Table 19), the GPM treated wound tissue seems to produce or display more glutathione peroxidase
activity then any another group of animals. This particular group exhibits as high as 2 fold of GPx level compare to the tissue of control animals.

**Ascorbic acid**

Though there was not much variation observed in the tissue ascorbate level among the treated groups, an obvious increase in the ascorbate level was seen in Group II, compare to its control, which is statistically significant (p<0.001).

**Superoxide dismutase**

The SOD level in the GPM treated wound tissue was found to show an increase to an extent of 40% to that of the control. Even amongst the other treated groups, the Group II animal specimens showed higher level of SOD activity (Table 19).

**Catalase**

The level of catalase, another antioxidant enzyme, in the wounded skin specimens of various treatment groups also records a relatively an increased activity in the GPM treated skin specimen compared with the other treatments as well as the control.
Lipid peroxidation

Oxidative damage to tissue or cell depends upon the defence system provided by the antioxidants. The resultant product of oxidative damage is identified by measuring the level of malondialdehyde. The results of Table 19 shows that the GPM treated wound tissue exhibited least MDA value, of all the other treatments, where it was observed to be about 2.6 fold less lipid peroxidation compare to the control.

5.5.4 Discussion

Cutaneous wound healing involves repair and regeneration of tissue. The healing process begins with the clotting of blood and is completed with remodeling of the cellular layers of the skin. Healing process, a natural body reaction to injury, initiates coagulation which controls excessive blood loss from the damaged vessels. The next stage of the healing process is inflammation and debridment of the wound followed by re-epithelialization, which includes proliferation, migration and differentiation of squamous epithelial cells of the epidermis. In the final stage of the healing process collagen deposition and remodeling occurs within the dermis (Chettibi and Ferguson 1997; Hj Baie and Sheikh, 2000). The involvement of each phase varies over a spectrum dependent largely on the type, location, milieu factors influencing the wound. Epithelialization is the process where keratinocytes migrate from the lower skin layer and divide. Contraction is the process where the wound contracts, narrowing or closing the wound. Matrix formation is facilitated by the movement of fibroblast in the wound area and
collagen is laid down over and throughout the amorphous material. (Rudolph 1979; Sunilkumar et al., 1998). Recent studies in the process of wound healing reveal that secretion of biologically active substances like growth factors, integrins, fibronectin and decon is inevitable for the initiation of healing process (Knighton et al., 1982; Dorminguez et al., 2002). Formation of granulation tissue, the primary step during the healing process is a complex event involving inflammatory cells, histocytes, plasma cells, mast cells and in particular in fibroblast that promote the growth of tissue by collagen production. Development and differentiation of granulation tissue are often influenced by endogenous and exogenous factors, which can promote the healing process (Tsuboi and Rifkin 1990).

In the present study, three different solvent extracts of *Gisekia pharnaceoides* designated as GPM, GPC, GPP applied topically on the wound and observed for its efficacy to promote the healing. Since the rate of wound healing is higher in GPM treated group followed by that of GPC and then the GPP, it is presumed that the crude methanolic extract of *Gisekia pharnaceoides* that was applied exogenously might have enhanced a centripetal migration of fibroblasts that advanced the wound contraction by 7 days compared to the control group. Many plant extracts and medicinal herbs have shown potential flavanoids, as main components of many extracts, act as powerful free radical scavengers (Tran et al., 1997; Berkercioglu et al., 1998). Role of antioxidants from plant extracts in wound healing has been reported earlier by Tran et al., (1996). In the present investigation, Kaempherol, a member of flavanoid family has been isolated in pure form
from methanolic extract and characterized (Chapter 3). This information provides authenticity that the enhanced wound healing in GPM treated group is due to Kaempferol. This apart, the preliminary phytochemical studies revealed the presence of tannins too, that are known to promote wound healing process mainly due to their astringent and antimicrobial property (Ya et al., 1988). Therefore the combined effect of flavanoid and tannins in the GPM would have triggered the wound contraction in 14 days. An observed 2 days delay of healing in GPC treated followed by 4 days delay in GPP treated groups suggests the absence or the presence of negligible quantity of flavanoids in GPC and GPP as they are the product of low polar solvents and therefore might not have extracted flavanoids into it.

The wound healing property of the plant extracts was evidenced by histological examination that revealed an increase in the level of collagen deposition and in the number of fibroblast cells compared to control. A more pronounced deposition of collagen fibers, well defined keratinocyte differentiation, presence of relatively moderate number of fibroblasts and absence of inflammatory cells observed in the GPM treated group support the claim that the combined effect of flavanoid and tannins accelerate the normal healing process and hence the increased rate of epithelialization compared to all other treatments. (Fig. 35)

Reactive oxygen species (ROS) such as superoxide radical (O$_2^-$), hydroxyl radicals (\'OH) or reactive nitrogen species (RNS) and nitric oxide (NO\') arise from inflammatory cells, which are strongly implicated in the
pathogenesis of several disease including chronic ulcer (Rojkind et al. 2002; Moseley et al., 2004 and Abd-El-Aleem et al., 2000). While ROS / RNS play an important role in the normal wound healing process by killing the invading microorganisms, the excessive overproduction of these species causes indiscriminate cellular damage, thus resulting in delayed healing. Flavanoids have been shown to possess free radical scavenging ability (Chen et al., 1990), therefore prevent the cell death (Jovanoic et al., 1996) by protecting them against lipid peroxidation (Dechameux et al., 1992). Studies on topical application of compounds with free radical scavenging properties on patients have been shown to significantly improve wound healing and protect tissue from oxidative damage (Martin 1996). The in vitro free radical scavenging ability of the different solvent extracts Gisekia pharnaceoides have been demonstrated in the early part of this chapter. These extracts are found to scavenge variety of ROS and RNS apart from DPPH' and ABTS'' radicals. In particular the NO radical is easily scavenged by the extracts and particularly the GPM was observed to be more active against all the radicals studied. This observation is fully reflected in the study of wound healing as well. This confirms the protective role of GPM, against the free radicals that is injurious to cell, even in the in vivo model.

To support this claim, the antioxidant status of the re-epithelialised tissue of different treatment of this study was assessed. The results of the enzymic and non enzymic antioxidant levels suggests that there is a significant increase in the antioxidant defence mechanism of treated animals compare to that of the control, and therefore showed better healing activity.
The phytochemical work of this investigation concluded that the extracts of *Gisekia pharnaceoides* contain certain bioactive compounds such as flavanoids and tannins that are found to be advantageous for various biological applications or therapies as discussed in earlier part of this report.

So scavenging effect might be one of the most important components of wound healing. Since the GPM treated wound tissue showed higher antioxidant levels, than that of other groups, a noticeable increase in the rate of healing was observed in this particular group (Group II). Therefore the enhanced rate of wound healing in the GPM treated group is due to the free radical scavenging action of flavanoid – tannin combine in addition to strengthening the invivo defence mechanism at the wound site. Many plant extracts and medicinal herbs have shown potent antioxidant activity. Flavanoids, the main components of many plants extracts act as powerful free radical scavengers (Tran *et al.*, 1997 and 1996, Berkercioglu *et al.*, 1998).

The protective role of GPM against the ROS is generally ascertained by monitoring the malandialdehyde (MDA), the lipid peroxidation product. A higher level of MDA in the control group specimen may be correlated with high level of unscavenged free radicals as against low level of MDA in the GPM treated group, attributing to the high order of free radical scavenging ability.
5.6 IN VITRO ANTHELMINTIC PROPERTY OF GISEKIA PHARNACEOIDES

5.6.1 Introduction

The prevalence of intestinal helmenthiasis is apparently very high, and on a global scale these infections cause severe health problems in man and domestic animals. More than one billion people are infected with *Ascaris lumbricoides*, and hundreds of millions are infected with hookworms and *Trichuris* (Guyatt and Evans, 1992). These infections cause intestinal disorders, discomfort and loss of productivity through direct or indirect interference with host nutrition and metabolism.

In livestock, gastrointestinal parasitic infections constitute a major obstacle to animal production all over the world, particularly in tropical and subtropical areas. The infections cause high economic losses in the form of impaired performance and reproduction, and sometimes significant weight losses and mortality rates (Fabiyi, 1986). A number of control measures to combat these infections are available, and several classes of modern synthetic anthelmintics have been shown to be very effective when used strategically in the right epidemiological context. However, increasing problems of development of resistance in helminths (Geerts and Dorny, 1995; Coles, 1997) against anthelmintics have led to the proposal of screening medicinal plants for their anthelmintic activities. These plants are known to provide a rich source of botanical anthelmintics (Satyavati *et al.*, 1976; Lewis and Elvin Lewis, 1977). A number of medicinal plants have been used to treat parasitic infections in man and animals (Nadkarni, 1954; Chopra *et al.*, 1956; Said,
Latex collected from young papaya fruits were shown to possess anthelmintic activity against *Ascaridia galli* infections in chickens (Mursof and He, 1991) and *Heligmosomoides polygyrus* in mice (Satrija et al., 1995). The use of *Calotropis procera* flowers as an anthelmintic in sheep have also been reported (Iqbal et al., 2005). Thus plants with anthelmintic properties offer an alternative to manufactured anthelmintics that is both sustainable and environmentally acceptable. Such plants could have a more important role in the future control of helminth infections in the tropics. With this view, the *Gisekia pharnaceoides* which also has the potential to serve as nutritional supplement (Chapter 4) is studied for its anthelmintic property against *Annelida*.

### 5.6.2 Material and Methods

(i) *Pheretima pothuma* (earth worm) nearly equal size (8±1cm) was collected from the local Horticulture Department.

(ii) Solution of GPP, GPC or GPM was prepared by dissolving each of extract substance in propylene glycol. Different concentration (25, 50, 100 and 200mg) of each of extract solution was prepared by diluting the stock solution, in propylene glycol, using normal saline. This serves as test drug.

(iii) The reference drugs- Piperazine citrate or albendazole was prepared by dissolving them in normal saline at a concentration of 15mg/ml.
(iv) A 10% propylene glycol in normal saline was used as experimental control – treatment.

(v) Saline was prepared and used to treat the normal control group.

**Experiment**

*Pheretima pothuma* was placed in petridish containing four different concentrations (25, 50, 100 and 200mg) each of GPP, GPC and GPM solutions. Each petridish was placed with 6 worms and observed for paralysis or death (Nargund 1999; Vigar 1984). The mean time for paralysis was noted when no movement of any sort could be observed, except when the worm was shaken vigorously; time of death of worm (min) was recorded after ascertaining that worms neither moved when shaken nor when given external stimuli. In the same manner piperazine citrate or albendazole was included as reference compound. These observations were compared with those of controls.

**5.6.3 Results**

The crude extracts samples which were used to evaluate anthelmintic activity, showed variable times at different concentrations and the mean time values were calculated for each parameter (Kulkarni 1999) and presented in Fig 41 and Table 20. The crude extract of GPP showed the significant anthelmintic effect causing death of the worm at all the concentrations but the time of death was different in each case. However, when we observed the
response of worms in case of paralysis, there was significant variation among the results produced by the different extracts at different concentration like 25, 50, 100 and 200 mg/ml. The GPP showed more significant effect on paralyzing the worms, in terms of paralysis time, at every concentration compared to that of GPC and GPM. Similar observations were made in the anthelmintic activity as well.

5.6.4 Discussion

The effect of extracts on the paralysis or helminthiasis of the worm, according to the results (Table 20) may be indicated as GPP > GPC > GPM. In particular, the GPP (Petroleum ether extract) exhibited an increased paralytic as well as helminthiatic effect over albendazole at the given experimental concentrateons (Table. 20). This may be due to the increased level of extraction of tannins in the GPP followed by GPC and then GPM, as the GPP is the low polar solvent and hence tend to extract more amount of tannin. The data presented in the Table. 20 and the observations made there of, lead to the conclusion that the different degree of helminthiasis of the different extracts are due to the level of tannins present in the compounds.

Tannins, the secondary metabolite, occur in several plants have been reported to show anthelmintic property by several investigators. (Yesilada et al., 1993; Sezik et al., 1997; Niezen et al., 1995; Khan and Diaz-Hernandez, 1999; Athnasiadou et al., 2001; Waller et al., 2001 ; Niezen et al., 1998). Tannins, the polyphenolic compounds, are shown to interfere with energy generation in helminth parasites by uncoupling oxidative phosphorylation
(Martin, 1997) or, bind to the glycoprotein on the cuticle of the parasite (Thompson and Geary, 1995), and cause death. Coming to the chemistry of nematode surface, it is a collagen rich extracellular matrix (ECM) providing protective cuticle that forms exoskeleton, and is critical for viability. The collagen is a class of proteins that are modified by a range co- and post-translational modification prior to assembly into higher order complexes or ECMs (Page and Winter 2003). The mammalian skin also consists largely of collagen in the form of fibrous bundles. In leather making industry, vegetable tannins are commonly used in the tanning operation of leather processing that imparts stability to collagen of skin matrix through its reactivity and hence make the collagen molecule aggregate into fibres. This results in the loss of flexibility in the collagen matrix and gain of mechanical property with improved resistance to thermal or microbial / enzymatic attack. Similar kind of reaction is expected to take place between the nematode cuticle (the earthworm) and the tannin of *Gisekia pharnaceoides*, possibly by linking through hydrogen bonding, as proposed in this study. This form of reactivity brings toughness in the skin and hence the worms become immobile and non functional leading to paralysis followed by death.

**STATISTICAL ANALYSES**

The data were subjected to analysis of variance and the significant differences among the mean compared with students `t' test using SPSS PC+ software.
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