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Nutritional Evaluation, *in vitro* Free Radical Scavenging and *in vivo* Anti-inflammatory Effects of *Gisekia pharnaceoides* and Identification of Kaempferol as a Nutraceutical Agent

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Research Article

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ABSTRACT

Aims: To analyse the nutritional profile and assess the antioxidant and antiinflammatory activities of *Gisekia pharnaceoides* wild plant used as traditional food source for dietary supplementation and to identify the bioactive compound as the nutraceutical agent.
Study design: In vitro and In vivo studies.
Place and Duration of Study: Department of Biotechnology, CSIR-Central Leather Research Institute, Chennai-600020, India and Sri Ramachandra College of Pharmacy, Sri Ramachandra University, Chennai-600116 between October 2005 and December 2007.
Methodology: Dry powder of the whole plant of *Gisekia pharnaceoides* was used for determining the nutritional parameters. Vitamins were estimated as per Indian Pharmacopoeia and United States Pharmacopoeia methods. Mineral composition was determined using Atomic absorption spectroscopy. Proximate analysis of plant power was carried out according to AOAC methods. Solvent extracts of the plant were assessed for free radical scavenging activities in vitro and antiinflammatory activity in vivo. UV, IR, NMR

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and LC-MS spectroscopy were used for identification of the bioactive compound.
Results: A markedly increased composition of vitamins such as thiamine, riboflavin, ascorbic acid and vitamin A and a wide range of minerals of metabolic importance have been estimated with high nutritive value. Additionally, the plant contained 9% protein and about 3% fat and carbohydrate content to an extent of 69%, of which 8% was crude fibre. All three extracts exhibited a high degree of free radical scavenging ability against DPPH radical, $NO^{\bullet}$, $OH^{\bullet}$, $ABTS^{\bullet+}$, $O_2^{\bullet -}$. The methanol fraction showed increased levels of antioxidant and anti-inflammatory activities compared to the other two extracts because of the presence of a flavonoid which was identified as kaempferol using UV, FTIR, NMR and LC-MS spectroscopy.
Conclusion: *Gisekia pharnaceoides* could therefore serve as a potential nutraceutical to prevent or inhibit the harmful oxidation process in human pathophysiology, and, is a high value nutritive source as a dietary supplement to prevent malnutrition especially in rural population. Therefore, we suggest the dietary intake of the plant for nutritional supplementation.

Keywords: Gisekia pharnaceoides; nutritive value; anti-inflammatory property; free radical scavenging ability; kaempferol;

1. INTRODUCTION

Gisekia pharnaceoides (GP) belongs to the *Molluginaceae* family and is an annual herb found predominantly in West African region and on the coastal sand dunes and river beds in India. This plant has been reported to be used in traditional medicine (Jeffrey, 1961) to treat asthma or applied topically to fight inflammation. The sap of the plant, sometimes, is used against warts. Lack of data on the chemical composition of wild plants has limited the prospects for their utilization (Viano et al., 1995). Wild plant species provide minerals, fibre, vitamins and essential fatty acids and enhance taste and colour in diets (Bianco et al., 1998). Recent study on the pharmacognosy of the leaf of *Gisekia pharnaceoides* revealed the presence of starch, proteins, oils and calcium oxalate while the preliminary phytochemical screening has been reported to show the presence of tannins, alkaloids, resins, cardiac glycosides, flavonoids and carbohydrates (Musa et al., 2006). These compounds in general contribute to several biological properties including hepatoprotective, anti-thrombotic, anti-inflammatory and anti-viral activities, many of which may be related, partially at least, to their antioxidant and free radical scavenging ability (Marcocci et al., 1994; Djeridane et al., 2006) and anti-inflammatory property (Moody et al., 2006). Generally, free radicals are essential for many cellular functions such as bacterial ingestion and phagocytosis. However, toxic species of radicals can cause oxidative damage and further, contribute towards aging and degenerative diseases of aging such as brain dysfunction, cataracts, cancers and cardiovascular diseases (Ames et al., 1993). Thus the usefulness of antioxidants in combating free radicals to protect cellular components in vivo against oxidative damage is warranted.

Similarly under inflammatory conditions, mammalian tissues respond 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 infiltration of blood cells causing oedema (Sobota et al., 2000). Therefore the usefulness of anti-inflammatory agents against such pathologies associated with oxidative insults in combating the free radicals to protect

cellular components against oxidative damage is essential. However, use of synthetic drug formulations in the treatment of these conditions has been reported to result in the incidence of a number of human diseases (Tapiero et al., 2002), while herbal constituents have mild contraindicative effects with a broad spectrum of biological activity. The present study is aimed to investigate the nutritional profile of the whole plant of GP for human consumption and as a dietary supplementation followed by extraction of the active principles and study its biological activities viz. antioxidant and anti-inflammatory properties.

2. MATERIALS AND METHODS

2.1 Chemicals

Carrageenan, diphenyl picryl hydrate (DPPH) and vitamin E (Sigma Chemicals), naphthyl ethylene diamine (Loba Chemie) and diclofence sodium (Novartis) were used in this study. All other chemicals or reagents used were of analytical grade.

2.2 Animals

Wistar albino rats 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, India. 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 feed, and tap water *ad libitum*. The experimental protocols of this study were approved by the Institutional Animal Ethics Committee (IAEC) of Committee for the Purpose of Control and Supervision on Experimentation on Animals (CPCSEA), vide sanction letter No IAEC 21 / XV / CLBMCP.

2.3 Plant Material

Dry powder of the whole plant of *Gisekia pharnaceoides* was used for determining the nutritional parameters. Three batches of the dry plant powder was prepared by pulverizing after drying under shade, the moisture content of which was about 12%.

2.4 Estimation of Vitamins

Estimation of vitamins A, C and riboflavin were carried out according to standard methods of Indian Pharmacopoeia (1996) and thiamine was estimated as per United States Pharmacopoeia (2000).

2.5 Elemental Analysis

The mineral composition of G*isekia pharnaceoides* was analyzed using Atomic Absorption Spectrophotometer Perkin Elmer model 2380. Quantification of elements such as Ca, Fe, Na, K, Mg, Cu, Mn, Zn and Cr were done according to the method of Hack (2000). The plant was dried at 80°C for 12 h and 1g of finely ground sample was treated with 5 ml of concentrated nitric acid and 2 ml of perchloric acid and digested by heating to obtain a final volume of 3–5 ml. The digest was filtered and the filter paper was washed with water and the filtrate was made up to known volume with deionized water. An aliquot of the filtrate was used for elemental analysis.

2.6 Proximate Analysis of Plant Powder

Proximate analysis of plant powder was carried out according to AOAC methods. Moisture and ash contents of the sample were determined by heating it at 110° respectively for 2–3 h. The fat was estimated using Soxhlet apparatus with petroleum ether as solvent. Nitrogen was determined by microkjeldahl method while crude fibre content was estimated by consecutive acid and alkali digestion of sample followed by washing, drying and then ashing at 600°C. The weight of ash free fibre was calculated.

2.7 Nutritive Value

Nutritive value of the dry powder of the plant was calculated based on the energy value available per kg of the macro nutrient. Normally, proteins, carbohydrates and fats yield 4.0, 4.0 and 9.0 Kcal of energy per g respectively. Therefore the nutritive value (NV) is calculated (Indrayan et al., 2005) as, [(4 × % protein) + (4 × % carbohydrate) + (9 × % fat)].

2.8 Preparation of the Plant Extract

The plant *Gisekia pharnaceoides* was collected afresh and shade dried between 20–30ºC. The plant was then powdered and used for extraction in different solvents. The dry powder of the whole plant was extracted in succession using petroleum ether, chloroform and then methanol. The individual extracts of *Gisekia pharnaceoides* (GP) in Petroleum ether (GPP); Chloroform (GPC); and in Methanol (GPM), were dried separately at 40ºC under reduced pressure using Buchi R-153 rotary evaporator. The extracts were then stored at 4ºC until further experimental use.

2.9 Qualitative Phytochemical Screening of Extracts

The different qualitative chemical tests for detection of various phytoconstituents such as alkaloids, proteins and amino acids, carbohydrates and glycosides, fixed oils and fats, saponins, phenolic compounds and flavonoids, phytosterols, volatile oil, gum and mucilages and tannins were performed according to standard methods (Harborne, 1998).

2.10 *In Vitro* Antioxidant Activities

2.10.1 DPPH scavenging assay

The free radical scavenging activity of the crude extracts of GP was examined in vitro by using DPPH radical according to the method followed by Yokozawa et al. (1998). Extracts of different concentrations, ranging from 25–800 µg ml^{-1}, were prepared and used in this study. This assay was based on the measurement of the scavenging ability of antioxidant substances towards the stable radical. 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), 0.05 ml of the extract in buffer and 1ml ethanol. The absorbance of the mixture was measured at 517 nm exactly 30 s after adding the crude extracts. The experiment was performed in triplicates and the extent of decolorization was measured as % scavenging. Parallely, a blank was made with all the reagents except the extracts.

2.10.2 Nitric oxide radical scavenging assay

Assay of nitric oxide radical ($NO^{\bullet}$) scavenging activity was performed according to the method of Marcocci et al. (1994). In this method, 5 µM sodium nitroprusside in 0.025 M phosphate buffer (pH 7.4), was incubated with different concentrations (25-800 µg ml^{-1}) of the extracts in the same buffer at 25°C for 5h. An aliquot of 0.5 ml of incubated solution was then diluted with 0.5 ml Griess reagent (prepared by mixing equal volume of 1% sulphanilamide in 2% phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride in water). The absorbance of chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with napthyl ethylene diamine was read at 546 nm. The control experiment was also carried out in a similar manner, using buffer in the place of extracts. The experiment was performed in triplicates and the extent of decolorization was measured as % inhibition of the nitric oxide free radical by the extracts

2.10.3 Hydroxyl radical ($OH^{\bullet}$) scavenging assay

The $OH^{\bullet}$ scavenging activity of the extracts was measured by studying the competitive reactivity between deoxyribose and the extracts for hydroxyl radicals, generated in the Fe^{3+} / ascorbate / EDTA / H_2O_2 system. The hydroxyl radicals attack deoxyribose, which eventually results in TBARS (Thiobarbituric acid reactive substance) formation. The reaction mixture contained 2.8 mM deoxyribose, 0.1 mM $FeCl_3$, 0.1 mM EDTA, 1 mM H_2O_2, 0.1 mM ascorbate, 20 mM KH_2PO_4 - KOH buffer (pH 7.4) and various concentrations (25-800 µgml^{-1}) of extracts in buffer or the reference drug in a final volume of 1 ml. The reaction mixture was incubated for 1 h at 37°C. Deoxyribose degradation was measured as TBARS at 532 nm (Ohkawa et al., 1979) and percentage scavenging was calculated from the absorbance values. Parallely, a control was made with all the reagents except the extracts.

2.10.4 $ABTS^{\bullet+}$ radical cation scavenging assay

The antioxidant capacity of each extract was evaluated by studying its ability to bleach the radical ($ABTS^{\bullet+}$). ABTS radical cation was produced by reaction of 7 mM ABTS solution with 2.45 mM ammonium persulfate, and the mixture was allowed to stand in dark at room temperature for 12–16 h before use. In this study, different concentrations (25–800 µg ml^{-1}) of the crude extract was added to 0.3 ml of ABTS solution and the final volume was made up to 1 ml with ethanol and the absorbance was read at 745 nm after mixing (Miller and Rice-Evans, 1997). The percentage scavenging was calculated from the absorbance values. Parallely, a blank was made with all the agents except the extracts.

2.10.5 Superoxide radical ($O_2^{\bullet-}$) scavenging assay

The assay was performed by using alkaline dimethyl sulphoxide (DMSO) according to the method of Henry et al. (1976). In this assay, potassium superoxide and dry DMSO were allowed to stand in contact for 24 h and the solution was then filtered immediately before use. A 200 µl of the filtrate was added to 2.8 ml 10 mM potassium phosphate buffer (pH 7.4) containing 56 µM nitroblue tetrazolium (NBT) and 10 µM EDTA. To this, crude extracts of GP (1 ml) of various concentrations (25–800 µg ml^{-1}) were added and the absorbance was recorded at 560 nm, against a control without extract in which pure DMSO was added instead of alkaline DMSO and the percentage scavenging was calculated from the absorbance values. 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.

2.11 Assay of Anti-Inflammatory Activity

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[a]) orally, as mentioned below. The test group of rats received a single dose of either 250 mg or 500 mg of crude extract per kg body weight. The animals were treated as follows:

Groups	Treatment
Group I	Control -1% SCMC [a] 10 ml kg^{-1} body weight
Group II	GPP 250 mg kg^{-1} body weight
Group III	GPP 500 mg kg^{-1} body weight
Group IV	GPC 250 mg kg^{-1} body weight
Group V	GPC 500 mg kg^{-1} body weight
Group VI	GPM 250 mg kg^{-1} body weight
Group VII	GPM 500 mg kg^{-1} body weight
Group VIII	Diclofenac sodium 5 mg kg^{-1} body weight

[a]*Sodium Carboxy Methyl Cellulose*

2.11.1 Induction of rat hind paw oedema

The carrageenan induced hind paw test 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 oedema of the injected and contra-lateral paws were measured at +1, 2, 3, 4 and 5 h after induction of inflammation using a plethysmometer. Results were expressed as percentage inhibition oedema, calculated according to the following formula (Garcia et al., 1996):

$$\text{PERCENTAGE INHIBITION} = \frac{[\text{CONTROL - TEST}]}{\text{CONTROL}}$$

2.12 Statistical Analysis

For studies on nutritive characteristics, all data are reported as Mean ± standard deviation (S.D) of three different experiments. For studies on biological activities, the statistical analyses were performed by the method of Dunnett and Tukey HSD tests with the level of significance at $p < 0.001$.

2.13 Separation and Characterization of Compounds

Each of the three extracts (concentrates) obtained by successive extraction using petroleum ether, chloroform and methanol was subjected to silica gel column chromatography to isolate the active principles. Column chromatographic fractions eluted at different intervals were resolved on TLC plate using appropriate mobile phase. Fractions showing similarity in band pattern on TLC were pooled together and concentrated in vacuum for further characterization studies. The purified fractions were characterized by using various spectral methods such as UV, IR, ^{1}H-NMR, ^{13}C-NMR and LC – Mass spectrometry.

3. RESULTS

3.1 Nutritional Characteristics of *Gisekia pharnaceoides*

The proximate compositions of the plant *Gisekia pharnaceoides*, in powder form, are reported in Table.1.

Table 1: Proximate analysis of *Gisekia pharnaceoides* powder

Sl. No.	Parameters	Content /100 g dry powder (Mean ± S.D of triplicates)
1.	Moisture	11.8 ± 0.2 g
2.	Protein	9.07 ± 0.02 g
3.	Carbohydrate	68.85 ± 0.02 g
4.	Fat	2.88 ± 0.02 g
5.	Fibre	8.1 ± 0.1 g
6.	Total ash	7.4 ± 0.3 g
7.	Nutritive energy	337.6 ± 0.05 cal

The shade dried plant showed a moisture content of 11.8 %, and this sample was taken for further analysis and the results are expressed on dry matter basis. The plant *Gisekia pharnaceoides* seems to have a wide variety in composition, with a crude protein content of >9 percent, while the fat content was one third of the protein level. The crude fibre content of whole plant in powder form was observed to be 8.0 % on moisture free basis. The total ash content of the plant was 7.4 g /100 g and the total carbohydrate was calculated to be about 68-70 %. The gross nutritive energy obtained from 100 g of the dry *Gisekia pharnaceoides* powder was calculated to be 338 kcal.

The powdered plant material was also analyzed for vitamins A, B1 (Thiamine), B2 (Riboflavin), and C (Ascorbic acid) and the results are presented in Table 2.

Table 2: Vitamin contents of *Gisekia pharnaceoides* powder

Sl. No	Name of the vitamin	Content/100g dry powder (Mean ± S.D of triplicates)
1.	Vitamin A	3026 ± 0.2 IU
2.	Vitamin B_1 (Thiamine)	0.07 ± 0.005 mg
3.	Vitamin B_2 (Riboflavin)	20.22 ± 0.01 mg
4.	Vitamin C (Ascorbic acid)	4.65 ± 0.05 mg

The wild plant in this study is observed to contain an appreciable amount of vitamins such as vitamin A, vitamin B1, vitamin B2 and vitamin C. The results showed that *Gisekia pharnaceoides* is a good source of vitamin A (3430 IU/100g).

The mineral contents of G*isekia pharnaceoides* are evaluated in Table.3. The availability of calcium in *Gisekia pharnaceoides* is the highest (410 mg/100g) of all the minerals. The level of magnesium (15 mg) available in the plant is higher than that of iron (9 mg). The copper content (0.26 mg) in *Gisekia pharnaceoides* represents to about 13% of the recommended daily requirement (2 mg). Chromium is found in traces in the plant.

Table 3: Mineral contents of *Gisekia pharnaceoides* powder

Sl. No.	Name of the element	Content mg/100 g dry powder (Mean ± S.D of triplicates)
1.	Calcium	410 ± 0.5
2.	Iron	9.15 ± 0.03
3.	Magnesium	14.74 ± 0.02
4.	Sodium	59.19 ± 0.01
5.	Potassium	192.88 ± 0.02
6.	Copper	0.26 ± 0.002
7.	Manganese	0.93 ± 0.015
8.	Zinc	10.59 ± 0.01
9.	Chromium	0.004 ± 0.0001

Apart from the major elements required for human metabolism, the micronutrients are also found to be present in this plant. The different nutritional parameters of some of the edible plants reported earlier by McCollum (1992) are presented in Table 4 for comparison.

Table 4: Protein (g/100g) mineral and ascorbic acid contents (mg/100 g) of some selected cultivated vegetables[a]

Species	Protein	K	Ca	Fe	Na	Mg	P	Vit C
Lettuce	1.6	264	68	1.4	9	11	25	18
Spinach	2.9	470	93	3.1	71	66	51	51
Parsley	2.2	727	219	6.2	45	14	63	172
Cabbage	1.2	233	49	0.4	20	21	29	47

[a]*The values shown in this table are cited from McCollum (1992)*

3.2 Phytochemical Screening

The dried whole plant powder of GP subjected to successive extraction in the order of petroleum ether, chloroform and methanol tested positive for the presence of various phytochemicals including tannins and flavonoids. The methanolic extract, however exceptionally showed the presence of alkaloids in addition to proteins and amino acids. Though all the extracts tested positive for flavonoids and tannins in their qualitative tests (data not presented), the quantity of these bioactive compounds present in a particular extract, may be different depending upon the polarity of the solvent. On this basis, the anti-oxidant and anti-inflammatory properties of the crude extracts have been discussed.

3.3 Diphenyl Picryl Hydrate (DPPH) Radical Scavenging Ability

The change in absorbance produced by reduced DPPH was used to evaluate the quenching ability of the crude extracts (GPP, GPC and GPM) to act as free radical scavengers. DPPH decolorization was increased by the presence of GPM, GPC or GPP in a concentration dependent manner with IC_{50} of 155, 195 and 260µg ml^{-1} respectively (Figure 1). Vitamin E that was used as the reference drug was also found to scavenge DPPH with IC_{50} of 12µg ml^{-1}.

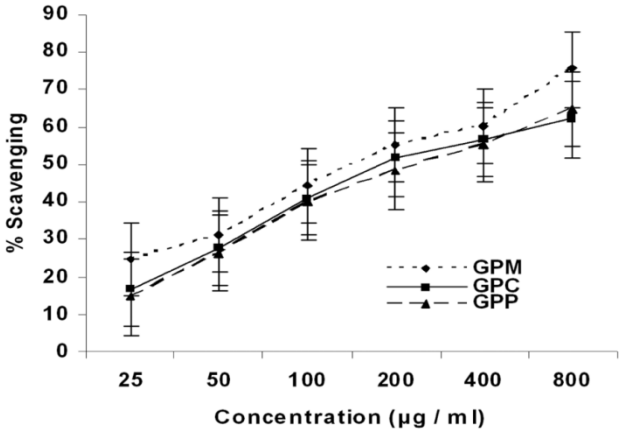

Figure 1: Percentage scavenging activity of plant extracts (GPM, GPC and GPP) towards DPPH radical

3.4 Nitric Oxide Radical Scavenging Activity

The overall efficacies of GPP, GPC and GPM on inhibition of nitrite and nitrate production are shown in Figure 2. Each sample was assayed at different concentrations ranging from 25–800 µg ml^{-1}. The individual activity of all the samples was compared with that of vitamin E. The decrease in absorbance in the presence of plant extract was measured as the $NO^{\bullet}$ inhibitory activity. GPM extract showed high order of nitric oxide scavenging activity followed by GPC and GPP with IC_{50} of 140, 175 and 250 µg ml^{-1} respectively. Vitamin E, the reference drug also showed nitric oxide radical scavenging activity with an IC_{50} of 23 µg ml^{-1}.

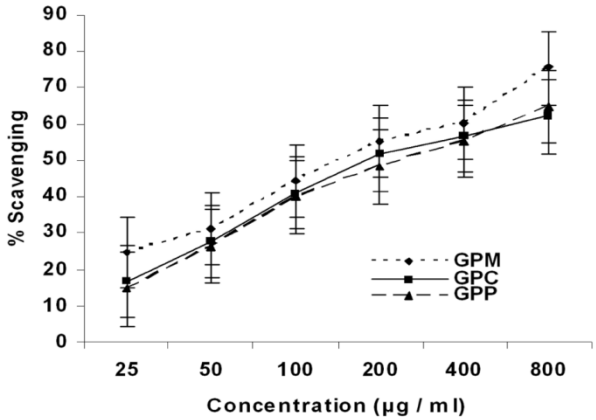

Figure 2: Percentage scavenging activity of plant extracts (GPM, GPC and GPP) towards nitric oxide radical

3.5 Hydroxyl Radical Scavenging Activity

The hydroxyl radical scavenging activity can often be calculated using deoxyribose assay. The hydroxyl radical produced by $FeCl_3$-EDTA-H_2O_2 system in the presence of ascorbate is competitively scavenged by ribose and the test compounds.

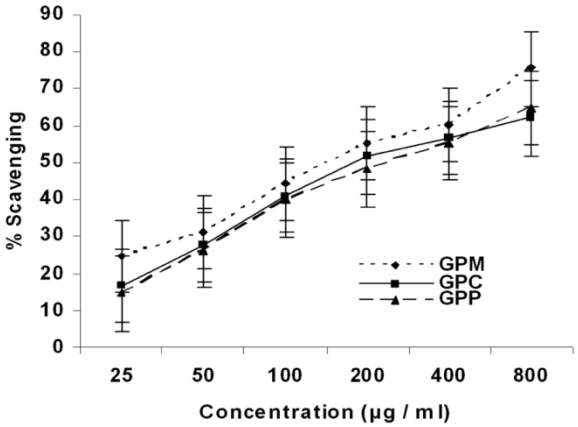

Figure 3: Percentage scavenging activity of plant extracts (GPM, GPC and GPP) towards hydroxyl radical

The radical that is not scavenged by the plant extract, in this study, attack the deoxyribose present in the reaction mixture and degrades it into a series of fragments, which react on heating with thiobarbituric acid at low pH to give a pink chromogen. Thus the scavenging activity towards hydroxyl radical of plant extract viz., GPP, GPC and GPM added to the reaction mixture was measured on the basis of the degradation of deoxyribose. All the three extracts, at all concentrations exhibited scavenging ability as could be seen in Figure 3 with an IC_{50} of 150, 180 and 250 µg ml^{-1} for GPM, GPC and GPP respectively, where as the IC_{50} for reference anti oxidant, Vitamin E, was found to be 12.5 µg ml^{-1}.

3.6 2,2-Azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) radical (ABTS) radical cation decolorization activity

The antioxidant activity of the different solvent extracts of GP was determined from the decolorization of $ABTS^{\bullet+}$ by measuring the reduction of radical cation as percentage of inhibition spectrophotometrically at 745 nm. Figure 4 illustrates the effect of concentration of the test compounds - GPP, GPC and GPM on the suppression of $ABTS^{\bullet+}$ radical scavenging activity. These results were compared with that of vitamin E which was used as reference antioxidant. On comparative basis, the methanolic extract (GPM) showed better activity in quenching $ABTS^{\bullet+}$ with an IC_{50} value of 155 µg ml^{-1} followed by chloroform extract (GPC) with 200 µg ml^{-1}, while the reference antioxidant Vitamin E showed an IC_{50} 10 µg ml^{-1}.

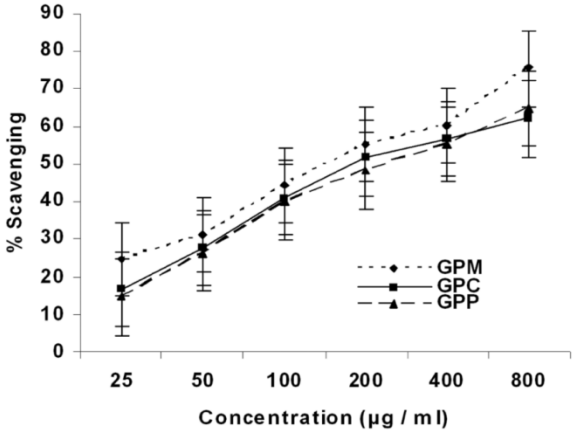

Figure 4: Percentage scavenging activity of plant extracts (GPM, GPC and GPP) towards ABTS radical cation

3.7 Superoxide Radical Scavenging Activity

The plant extract, added to the reaction mixture was capable of reacting with $O_2^{\bullet-}$ competitively and inhibit the production of formazan. The superoxide anion scavenging ability of the crude extracts of *Gisekia pharnaceoides* is illustrated in Figure 5. The test samples of this study showed superoxide radical scavenging activity with an IC_{50} ranging from 200–300 µg ml^{-1} compared to 13.5 µg ml^{-1} for vitamin E, the reference material.

3.8 Anti-Inflammatory Activity

The results of average paw volume and percentage of inhibition by different extracts and the reference drug obtained in the carrageenan induced rat hind paw oedema test are presented in Table 5. For the Control (untreated) group the injection of carrageenan caused localized oedema well in advance compared to other groups. In this untreated group, the swelling increased progressively to a maximum volume at 3 h after the carrageenan injection.

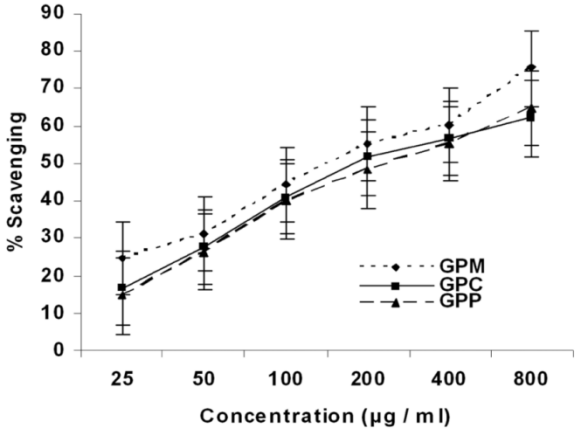

Figure 5: Percentage scavenging activity of plant extracts (GPM, GPC and GPP) towards superoxide radical

The rats pre-treated with the crude extracts of *Gisekia pharnaceoides* had significant reduction in the oedema volume on 3 h post dosing, at all dose levels used. The effect of GPM administration in controlling the paw oedema was almost comparable to that of the reference drug (Table 5). An increase in the volume of paw oedema was observed up to 3 h in all the groups, and thereafter it decreased. GPM at a dose level of 500 mg kg^{-1} body weight showed highest percentage inhibition (73.68%) of oedema at 3 h, 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 GPM kg^{-1} body weight dose was observed to be 56.5 and 73.68 % respectively.

3.9 Physical Characteristics of Isolated Compounds

Two compounds isolated in a detectable band size on thin layer chromatography (TLC) plate, from GPP and GPM by column chromatography were designated as compound I and II respectively. The compound I (of GPP) was white in colour and the melting point was found to be 68°C. It showed an R_f value of 0.62 on TLC plate in the solvent system of chloroform (100%).

The spot turned light green colour after spraying with 10% (vv^{-1}) H_2SO_4 followed by heating in hot air oven at 110°C for 10 min. It gave blue colour in UV light at 254 nm but not with ferric chloride indicating the absence of phenolic hydroxyl group. The compound was soluble in chloroform and acetone. Other spectral characterization methods confirmed (data not presented) that the compound I is an aliphatic hydrocarbon (dotriocontane M.W 450 Da) which has no biological significance and therefore not discussed further in this paper. On the other hand, the compound II (of GPM) was yellow in colour with a melting point of 278°C. It showed an R_f value 0.67 on TLC plate in the solvent system of chloroform 100%. The spot turned light green colour after spraying with 10% (vv^{-1}) H_2SO_4 and heating in hot air oven at 110°C for 10 min. It gave yellow colour in UV light at 254nm which melted at 278° C and produced a blue colour with ferric chloride. The compound was soluble in methanol and acetone.

Table 5: Anti inflammatory activity of the *Gisekia pharnaceoides* plant extracts by Carrageenan induced paw oedema in rats

Carrageenan induced paw oedema (volume in ml) in rats at different periods				
Group	**1 h**	**2 h**	**3 h**	**4 h**
Control	0.48±0.0037	0.556±0.0042	0.57±0.0033	0.55±0.0037
GPP 250 mg kg^{-1}	0.21±0.0033*	0.23±0.0037*	0.23±0.0048*	0.22±0.0037*
GPP 500mg kg^{-1}	0.24±0.0036*	0.23±0.0043*	0.21±0.0033*	0.20±0.0036*
GPC 250 mg kg^{-1}	0.32±0.0031*	0.33±0.0037*	0.27±0.0036*	0.24±0.0036*
GPC 500 mg kg^{-1}	0.27±0.0058*	0.26±0.0048*	0.24±0.0031*	0.23±0.0048*
GPM 250mg kg^{-1}	0.32±0.0048*	0.29±0.0047*	0.24±0.0048*	0.22±0.0037*
GPM 500mg kg^{-1}	0.28±0.0031*	0.24±0.0047*	0.15±0.0036*	0.14±0.0036*
Diclofenac sodium 5 mg kg^{-1}	0.15±0.0036*	0.14±0.0037*	0.12±0.0026*	0.11±0.0036*

*Values are expressed as Mean ± SEM of six experiments (six animals) in each Group. Statistical analysis was carried out by ANOVA followed by Dunnet's 't' test. Comparisons were made between control vs drug-treated groups; * p<0.001.*

3.10 Spectroscopic Analyses of GPM Compound

The presence of any conjugation in the GPM–compound was identified from UV–vis spectrum. Thus, GPM–compound showed an absorption maximum at 325 nm, showing the presence of enone chromophore in the compound.

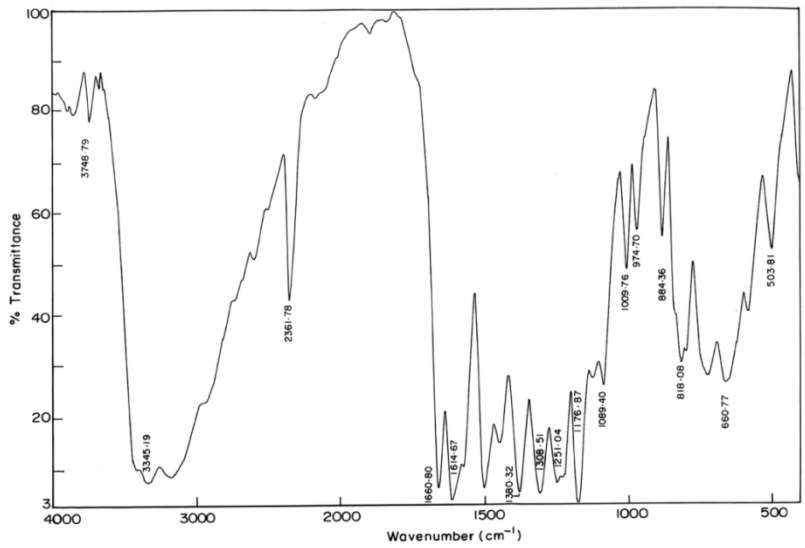

Figure 6: FT-IR Spectrum of GPM Compound

The presence of functional groups was established from FTIR-spectrum. The FTIR spectrum of GPM compound (Figure 6) showed absorption peak for hydroxyl or phenolic -OH at 3345 cm^{-1} and a broad O-H stretching absorption at 2361 cm^{-1}. C=C stretching band of vinyl group, conjugation with phenyl group were observed at 1660 $cm^{-1}$and 1614 cm^{-1}, respectively. Absorption band of C-O-H bending at 1380 cm^{-1}, C-O-H in plane bending at 1308 cm^{-1}, C-O stretching at 1251 cm^{-1}, C-O-C stretching at 1176 cm^{-1}, =C-H wagging absorption at 1009 cm^{-1} and 974 cm^{-1}, out of plane C-H bending at 818 cm^{-1} and broad hydroxyl bond, out of plane O-H bending at 660 cm^{-1} were also observed.

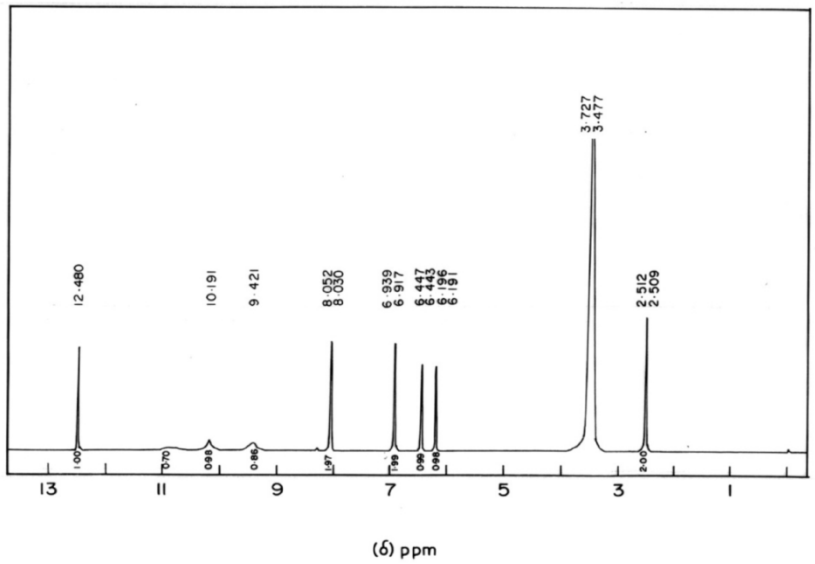

Figure 7: ^{1}HNuclear Magnetic Resonance spectrum of GPM compound isolated from *Gisekia pharnaceoides*

The key structural information of GPM compound (Figure 7) was arrived from ^{1}H-NMR spectrum. ^{1}H-NMR (300MHz, DMSO – d_6) δ : 6.19 (d, J =1.5Hz, 1H), 6.44 (d, J=1.2Hz, 1H), 6.91 (d, J=6.6Hz, 2H), 8.03 (d, J =6.6Hz, 2H), 9.42 (br s, 1H), 10.19 (br s, 1H), 11.00 (br s, 1H) and 12.48(s, 1H). The three hydroxyl protons were observed as broad singlets at δ 9.42, 10.19 and 11.00 ppm, respectively. The aromatic protons were observed in the region between δ 6.19–12.48 ppm. The detailed ^{1}H-NMR spectral study indicated the presence aromatic protons.

The ^{13}C-NMR (75MHz) spectrum showed following peaks: δ 93.96, 98.67, 103.50, 115.90, 122.14, 129.97, 136.10, 147.27, 156.64, 159.62, 161.73, 164.33 and 176.33 ppm (Figure 8). Aromatic and olefinic carbons were observed in the region δ 93.95–164.33 ppm and the carboxyl carbon was observed at δ 176.33 ppm.

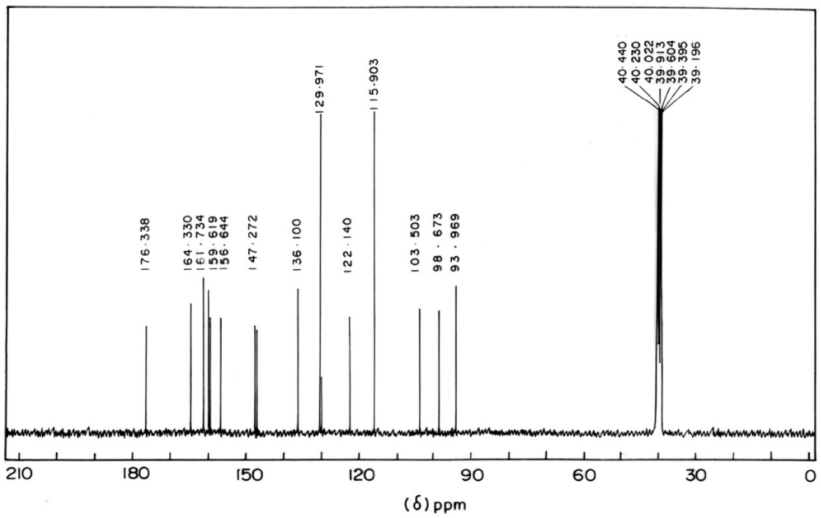

Figure 8: ^{13}C Nuclear Magnetic Resonance spectrum of GPM Compound

LC – Mass of GPM compound showed a single peak at retention time 0.622 min (Figure 9) suggesting the presence of a single compound in pure form.

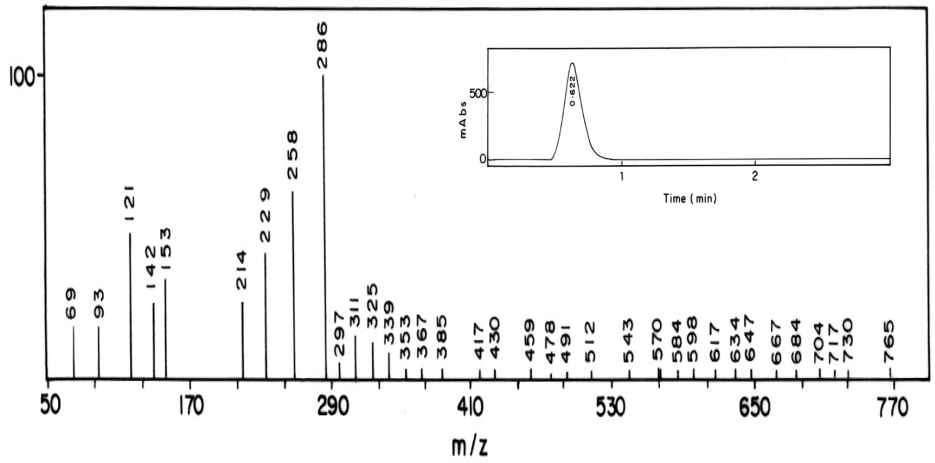

Figure 9: LC-Mass spectrum of GPM Compound, LC chromatogram

The mass spectrum of this compound showed a molecular ion peak at m/z 286. Based on the detailed spectroscopic study the structure of the GPM compound was confirmed as kaempferol (Figure 10).

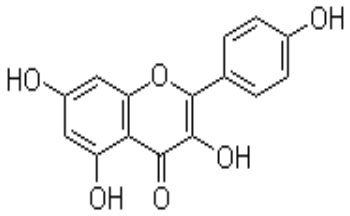

Figure 10: Chemical structure of kaempferol

4. DISCUSSION

4.1 Nutritive Profile of *Gisekia pharnaceoides*

Appropriate intake of nutrients is essential for normal organ development and function, repair of body tissues and combating stress and diseases. Most reports on unconventional crops indicate that they could be good sources of nutrients, and many have the potential of broadening the food base of human species (Okigbo, 1977). Hence, a need to preserve new plant resources in human nutrition has been emphasized (Williams, 1993). The herb chosen for the study in the present investigation belongs to a wild plant type and is being used for edible purpose in some interior parts of India. In this study, the nutritive value of *Gisekia pharnaceoides* was determined for the first time and reported in this paper. The protein content, >9% as seen from Table.1, shows that *Gisekia pharnaceoides* is a rich source of protein, compared to other edible plants reported by Coulter et al. (1988). The commonly used vegetables such as lettuce, spinach, parsley and cabbage contain protein of 1-3 % range. *Gisekia pharnaceoides*, would substantially supplement the protein to human diet, for which the Recommended Daily Allowance (RDA) of protein is 50 g/day for a normal adult. A high carbohydrate content of 68% found in *Gisekia pharnaceoides* might have been contributed by soluble carbohydrates and the crude fibre. The observed 8.1% crude fibre content in the *Gisekia pharnaceoides* (Table 1) may be the combination of hemicellulose, cellulose and lignin. The RDA of carbohydrate for normal adult is 300 g/day; whereas, for fibre it is 25 g/day.

4.2 Vitamin Analysis of *Gisekia pharnaceoides*

The wild plant of this study is observed to contain an appreciable amount of vitamins such as vitamin A, vitamin B1, vitamin B2 and vitamin C. The results showed that *Gisekia pharnaceoides* is a good source of vitamin A (3430 IU/100g) and riboflavin (>20 mg/100g) Riboflavin has been reported to be photosensitive and therefore a 25% loss in the vitamin content in alfa- alfa when exposed to bright sun shine for 48h was observed (Hunt et al., 1980). Hence, *Gisekia pharnaceoides* was processed in shade and the powdered material thus obtained was analyzed for riboflavin content. Natural ascorbic acid is vital for the body performance (Okwu, 2004) and the plant contained about 4.65 mg/100g of this vitamin. This study therefore provides nutritional basis of *Gisekia pharnaceoides* for its dietary use in the prevention of vitamin deficiency disease.

4.3 Mineral Constituents of *Gisekia pharnaceoides*

Ash content represents the total mineral content, which was found to be 7.4 % for *Gisekia pharnaceoides* and is part of the proximate analysis for nutritional evaluation. The elemental analysis data suggested that the plant is rich in minerals of nutritional importance. The availability of calcium in *Gisekia pharnaceoides* is the highest (410 mg) followed by potassium content (192 mg) and therefore the plant could be one of the best dietary sources of the macronutrients. Iron (9.15 mg) is well known for the production of hemoglobin to carry oxygen throughout the body. Magnesium (14.74 mg) is required in the plasma and extra cellular fluid to maintain osmotic equilibrium. Copper is a component of enzyme systems such as cytochrome oxidase and represents about 13% (0.26 mg) of RDA (2 mg). Chromium is essential in carbohydrate and lipid metabolism and is found to be in trace amounts. Zinc content (10.59 mg) is equivalent to 70% of its daily requirement and acts as a multifunctional

nutrient. Manganese, which is essential for hemoglobin formation is relatively higher (0.93 mg) than copper.

4.4 Antioxidant Profile of *Gisekia pharnaceoides*

Chemical compounds that exhibit antioxidant properties are widely present in a large number of aromatic and other medicinal plants (Mathew and Abraham, 2006). These compounds including alkaloids and flavonoids have been reported to be used as basic medicinal agents for their analgesic, antispasmodic and bactericidal effects (Okwu and Okwu, 2004). Flavonoids, on the other hand are potent water-soluble antioxidants and free radical scavengers, which prevent oxidative cell damage, have strong anticancer activity (Salah et al., 1995). Therefore the flavonoid containing GP can also be used to prevent the oxidative insult and the resultant metabolic disorder in the cells.

Polyphenols are broadly considered beneficial for human health because they are capable of scavenging free radicals and reactive oxygen species (Rice-Evans et al., 1996). Hence the in vitro scavenging efficiency of the crude extracts of GP was successfully tested against different free radicals in vitro. The phytochemical screening of the different crude extracts of GP revealed the presence of different groups of compounds of biological importance. Oxidative stress has been implicated in the pathology of many diseases including diabetes, cardiovascular disease, inflammatory conditions, cancer and aging. Antioxidants may offer resistance against oxidative stress by scavenging the free radicals, inhibiting lipid peroxidation and by many other mechanisms and thus prevent diseases (Youdim and Joseph, 2001).

DPPH assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical DPPH. The effect of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen donating ability. DPPH radicals react with the reducing agent, the electron becomes paired, and the solution loses color stoichiometrically depending on the number of electrons taken up (Shirwaikar et al., 2004). It is therefore understood from the results that GP reduces the DPPH radical to the corresponding hydrazine when it reacts with hydrogen donors in the antioxidants of the plant extract. Of the three solvent extracts studied, GPM exhibited increased DPPH radical scavenging activity. Similarly methanol extracts of four medicinal plants in the Philippine region exhibited DPPH scavenging abilities as recently reported by Peteros and Uy (2010).

Nitric oxide is a free radical produced in mammalian cells, involved in the regulation of various physiological processes. However, excess production of $NO^{\bullet}$ is associated with several diseases such as cancer (Nguyen et al., 1992), diabetes (Corbett and McDaniel, 1992), atherosclerosis, multiple sclerosis (Parkinson et al., 1997) and arthritis and in inflammations (Ohshima et al., 2003). In the recent years, herbal drugs having nitric oxide radical scavenging property are gaining importance (Rao et al., 2006). In the present study the nitrite produced by the incubation of sodium nitro prusside in standard phosphate buffer at 25°C was reduced by the extracts of Gisekia pharnaceoides. This may be due to the presence of antioxidant principles in the extracts which compete with oxygen to react with nitric oxide and thereby inhibit the generation of more deleterious products such as nitrous anhydride (N_2O_3) and perhydroxy nitrite ($ONOO^-$).

Ferrous salts can react with hydrogen peroxide and form hydroxyl radical via Fenton's reaction. The iron required for this reaction in a biological system is obtained either from the

pool of iron or the heme-containing-proteins (Cotran et al., 1999). The hydroxyl radical ($OH^{\bullet}$) thus produced may attack the sugar of deoxyribonucleic acid (DNA) causing ribose fragmentation, base loss followed by DNA strand breakage (Kaneko et al., 1997). The generation of $OH^{\bullet}$ in the Fenton reaction is due to the presence of iron ions. When the Fe^{2+} / Fe^{3+} redox couple is bound by certain chelators, the $OH^{\bullet}$ formation is prevented. As observed from the results the compound(s) of crude extracts seem to act as the chelators of iron ions and thereby preventing the formation of radical, though the extracts were not directly involved in the $OH^{\bullet}$ scavenging. Thus the results indicate that extracts of *Gisekia pharnaceoides* play a major role in the inhibition of ribose fragmentation.

The ABTS chemistry involves direct generation of ABTS radical mono cation with no involvement of any intermediary radical. It is a decolorization reaction and thus the radical cation assay is performed prior to addition of antioxidant test system, rather than the generation of the radical to occur continuously in the presence of antioxidants. The results obtained indicate the potential activity of the extract either by inhibiting the formation of, or scavenging, the $ABTS^{\bullet+}$ radical since both the inhibiting and scavenging properties of antioxidants towards $ABTS^{\bullet+}$ radicals, have been reported earlier (Salah et al., 1995).

Superoxides are produced from molecular oxygen by the oxidative enzymes of human system as well as via non enzymatic reaction such as autooxidation by catecholamines (Hemnani and Parihar, 1998). Superoxide anion is the first reduction product of oxygen, measured in terms of inhibition of generation of $O_2^{\bullet-}$ and superoxide dismutase catalyzes the dismutation of the highly reactive superoxide anion to oxygen and hydrogen peroxide (Kamalakkannan and Prince, 2003). The various phytocompounds such as tannins, polyphenols and flavonoids are reported to have antioxidant properties (Yokozawa et al., 1998;Brand-Williams et al., 1995) and hence, the compounds of *Gisekia pharnaceoides* that are observed in the preliminary phytochemical screening, may be responsible for the scavenging of superoxide, generated by potassium superoxide-alkaline DMSO system, in addition to other reactive species studied. The overall superoxide radical scavenging activity of the various extracts viz. GPM, GPC and GPP suggest that 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, compared to high polar solvents like chloroform and methanol.

The phytochemical screening of the extracts revealed the presence of flavonoids as well as tannins and other compounds. These compounds have been shown to posses various biological properties related to anti oxidant mechanisms (Adedapo et al., 2009). Therefore the antioxidant property exhibited by the crude extract against the radicals studied might be due to the flavonoids and tannins. Such observation has been made earlier by investigators for methanolic extract of other plants (Wang et al., 1996). Since the free radical scavenging property of the extracts against DPPH, $NO^{\bullet}$, $OH^{\bullet}$, $O_2^{\bullet-}$ and $ABTS^{\bullet+}$ radicals is confirmed in vitro, the *Gisekia pharnaceoides* plant as such, by all means should also serve as a potential antioxidant in vivo. In the 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 antioxidant rich food is added to the diet. Since Gisekia pharmaceoides is known to be an edible plant in certain regions, and is found to be an effective free radical scavenger in this study, it can serve as a potential antioxidant diet for those in the advancement of aging or age related disorders and diseases.

4.5 Anti-inflammatory Profile of *Gisekia pharnaceoides*

The anti-inflammatory effect of the different solvent extracts viz. GPP, GPC and GPM was also investigated using carrageenan as inflammatory agent, owing to its sensitivity in detecting orally active anti-inflammatory agents particularly in the acute phase of inflammation (DiRosa et al., 1971).

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 et al., 1969); whereas, 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 E_2 (PGE_2) have been noted in carrageenan challenged animals (Salvemini 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 immunomodulatory effect on various inflammatory responses, though the exact mechanism is yet to be understood. According to the results, the different extracts were shown to possess dose dependent degrees of anti-inflammatory activities. The mechanism of antiinflammatory action of plant flavonoids has been described by Kim et al. (2004).

The variation in the anti-inflammatory ability among the crude extracts of *Gisekia pharnaceoides* may be due to the compositional variation of the individual solvent extract. For instance, the anti-inflammatory activity of coumarins in 12-*O*-tetradecanoylphorbol-13-acetate (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 be possibly due to their ability to inhibit reactive oxygen species. All the three solvent extracts were tested positive for phenolic compounds including flavonoids as well as tannins and therefore exhibited antioxidant and anti-inflammatory activities to an extent depending upon the nature and concentration of bioactive compound present. Similar to our findings, methanolic extracts of 12 Indian medicinal plants exhibited active phytoconstituents than aqueous extracts (Parekh and Chanda, 2007).

The flavonoid isolated in pure form from the methanolic crude extract (GPM) of this study and characterized by FTIR, NMR and LC-Mass spectroscopy should be responsible for antioxidant and anti-inflammatory activities, in addition to other phytocompounds such as tannins. A recent report on *Celtis Africana* plant also revealed the effective scavenging of ABTS and DPPH radicals by the methanolic extract of leaves and stems (Adedapo et al., 2009). This property in turn would have resulted in the inhibition of lipid peroxidation and subsequently the inflammation that is noticed in the form of swelling.

Accumulating evidence indicates that excessive production of $NO^{\bullet}$ plays a pathogenic role in both acute and chronic inflammation. Moreover, $NO^{\bullet}$ is responsible for the vasodilatation, increase in vascular permeability, oedema formation and synthesis of prostaglandins at the site of inflammation (Grisham et al., 1999). Manipulation of NO free radical can be a potential and promising therapeutic area in treating inflammation. Approaches being currently used for inflammatory disorders include $NO^{\bullet}$ scavengeing as well as $NO^{\bullet}$ inhibition (Mittal et al., 2003). Since all the test drugs (GPP, GPC and GPM) exhibited significant in vitro $NO^{\bullet}$ free radical scavenging activity the same may be, in part, attributed to the observed

anti-inflammatory effect of these test drugs. The increased rate of inhibition up to 3 h exhibited by GPM suggest that its constituent is actively involved in the reactions of first phase as well as in 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. Whereas, the response recorded by the other extracts (GPP and GPC) were much slower compared to that of GPM and more particularly in the second phase (after 2h). However, the pretreatment by GPP and GPC seemed to have strengthened the defense system and thereby the paw oedema was reduced to as low as 58% in the initial stage, and thereafter does not seem to have participated in the anti-inflammatory response.

4.6 Characterization of GPM Compound by Spectroscopic Methods

The GPM compound eluted from column chromatography using chloroform: methanol (1:9) as eluent, exhibited a single peak in UV–vis spectrum. It was yellow colour in UV light which melted at 278° C which produced a blue colour with ferric chloride, indicating the presence of phenolic hydroxyl group. The purity of GPM compound was confirmed with LC – Mass which showed a single peak with 100% purity. The mass spectrum of the compound revealed the molecular weight of m/z = 286 (M^+).

In the IR spectrum of compound, the absorption peak at 3345 cm^{-1} represent the free hydroxyl group of alcohols or phenols. The presence of C=C stretching of vinyl group in conjugation with phenyl group absorb at 1660 cm^{-1}, 1614 cm^{-1} respectively. The presence of C-O-H bending is seen by absorption at 1308 cm^{-1} where as the C-OH in plane bending at 1308 cm^{-1}. The peaks at 1251 cm^{-1} and 1176 cm^{-1} indicate the presence of C-O stretching and C-O-C stretching, respectively. The two bands arising from =C-H wagging of aromatic alkenes occur near 1009 cm^{-1}, 974 cm^{-1}, respectively. Position of absorption of the out-of-plane band is characteristics of the number of adjacent H atoms on the un-substituted benzene ring and appears at 900-675 cm^{-1} which is seen at 818 cm^{-1} in the GPM compound. The ^{1}H-NMR spectral study indicated the presence of aromatic protons in the range of δ 6.19–8.48 ppm and hydroxyl protons as broad singlets at δ 9.42, 10.19 and 11.00 ppm, respectively. The ^{13}C-NMR spectrum provided additional information to deduce the GPM compound. All the data discussed above clearly supported the structure of GPM compound to be kaempferol.

5. CONCLUSION

Gisekia pharnaceoides, a wild plant has been evaluated for its compositional and nutritional properties. It is a high value nutritive source and can be used as a dietary supplement to prevent malnutrition especially in rural population. Therefore, we suggest the dietary intake of the plant for nutritional supplementation. The results have shown good correlation between the anti-inflammatory and antioxidant activities of the *Gisekia pharnaceoides*. It is therefore speculated that the GPM, due to its kaempferol content, was able to interfere in the release of histamine, serotonin and bradykinin as well as in the $NO^{\bullet}$ scavenging and /or inhibiting the formation of other injurious products of $NO^{\bullet}$ and thus affect $NO^{\bullet}$ induced inflammation either directly or through prostaglandin pathway. The investigation also supports the ancestral perception of using this plant in treating the degenerative diseases associated with the excess production of reactive oxygen or reactive nitrogen species. The identification of the active principle as kaempferol can further be investigated to chemically synthesize the compound for drug formulation approaches as a neutraceutical supplement in the pharmaceutical market.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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