4.1) MATERIALS AND METHODS

4.1.1) Plant material

Dried roots of *Glycyrrhiza glabra* were purchased from the local stores. The fresh leaves of *Nelumbo nucifera* were collected from the botanical garden of Botany department, The M. S. University of Baroda, Vadodara. All the plant materials were identified by Botany Department, The M. S. University of Baroda. The voucher specimens of the herbs have been deposited in the Pharmacy department, The M.S. University of Baroda. The *Prunella vulgaris* herb extract and *Zizyphus jujube* fruits extract were provided from Amsar pvt.ltd, Indore, M.P. India as a gift samples with certificate of Analysis. (COA)

4.1.2) Preparation of powdered material

The selected plant materials were collected, cleaned to remove any adhering material and then dried in shade. The large dried plant parts were then subjected to size reduction to coarse powder and used for further studies.

4.1.3) Standardization of the plant materials as per the WHO guidelines [1]

4.1.3.1) Macroscopic evaluation [2]

The size, colour, surface characteristics, texture, fracture characteristics and odour of the crude drugs were studied and compared with the standard monographs.

4.1.3.2) Microscopic evaluation [3]

The intact crude drugs as well as powdered drugs were studied under the microscope to analyze the cellular characteristics of the drugs.

4.1.3.2.1) Study of Transverse section

The T. S. of selected drug samples were taken in a test tube and 5 % potassium hydroxide in methanol was added so that the sample remained submerged. The samples were boiled for few minutes. Transverse sections of the drugs were taken in a watch glass containing water with the help of a brush. The sections were then transferred to a watch glass containing phloroglucinol-hydrochloric acid and allowed to stain for 2-3 minutes. The sections were again transferred to watch glasses containing water, so as the excess stain was washed away. The sections were then placed on clean glass micro-slides, with the help of brush. Few drops of water were

added and a clean cover-slip was placed on the slide. The slides were mounted for study on the microscope.

4.1.3.2.2) Study of Powder characteristics

The microscopic structures of powdered drugs were also studied using the slides prepared by above method using powdered drugs in place of sections.

4.1.3.3) Determination of ash [2]

After ignition, the remaining ash of medicinal plant materials was determined by in terms total ash, acid-insoluble ash and water-soluble ash.

4.1.3.3.1) Total ash

About 2 gm of the ground drug was weighed accurately in a previously ignited and tarred crucible. The material was spread in an even layer and ignited by gradually increasing the heat to 500 to 600°C until it was white in colour that indicates the absence of carbon. Then ash was cooled at room temperature and weighed. The total ash was calculated in % of air-dried material.

4.1.3.3.2) Acid-insoluble ash

About 25 ml 70% Hydrochloric acid (HCl) was added to the total ash and then covered with a watch glass. After boiling the mixture for 5 min, the insoluble matter was collected on an ash less filter paper and transferred to the crucible and ignited to constant weight. The residue was weighed and the acid-insoluble ash was calculated in % of air-dried material.

4.1.3.3.3) Water-soluble ash

The mixture containing 25 ml of water and total ash was boiled for 5 min. The insoluble matter was collected on an ash less filter paper. The crucible was ignited to constant weight. The weight of the residue was subtracted from the weight of total ash and the content of water-soluble ash in % of air-dried material was calculated.

4.1.3.4) Determination of Extractable matter [2]

The amount of the active constituents extracted with different solvents from a given amount of plant material was determined. The different solvents are used according to its polarity such as petroleum ether, toluene, chloroform, ethyl acetate, acetone, methanol and water. The method of hot extraction was used for this purpose. About 4.0 gm of coarsely powdered material was accurately weighed in a glass-stoppered

conical flask. 100ml of the specified solvent was added and the final weight was recorded as the total weight. The flask was kept at room temperature for 1 hour after proper agitation. A reflux condenser was attached to the flask and boiled gently for 1 hour, cooled and weighed. The total weight was readjusted to the original weight with the solvent specified. It was then filtered rapidly through a dry filter. 25 ml of the filtrate was transferred to a tarred flat-bottomed dish and evaporated to dryness on a water-bath. The extract was dried at 105°C for 6 hours, cooled and then weighed. The content of the extractable matter in % of air-dried material was calculated.

4.1.3.5) Determination of water and volatile matter [2]

About 2 gm of plant material was accurately weighed in a previously dried and tarred flat weighing bottle. The sample was dried in an oven at 100-105°C until there was no difference in the consecutive weighing. The loss of weight in % of air-dried material was calculated.

4.1.3.6) Determination of pesticide residues [4]

The presence of organo-phosphorus and organo-chlorinated pesticides was analyzed using TLC studies. An aqueous extract of the formulations were prepared and spotted on silica gel G plates. The following solvent systems and detecting reagents were then sprayed. The observations were recorded. Two mobile phases were used, DMF: Ether (4:6) and Methyl cyclohexane. The detecting reagent for organo chlorinated pesticides was tetra bromo phenolphthalein, and for organophosphorus pesticides was 0.5% Silver nitrate in water and acetone.

4.1.3.7) Determination of heavy metals [2]

Heavy metals like arsenic, mercury, lead and cadmium were analyzed using atomic absorption spectrophotometer in the ash of the powdered drug material. The elements detected were quantified by using standard calibration plots and expressed in PPM.

4.1.3.8) Determination of microorganisms [2]

The plant materials were analyzed for bio-burden present as per the reported methods. The tests were performed to determine the amount of aerobic bacteria and presence of yeasts and moulds, *E.coli, Enterobacteria* and *Salmonellae* using the reported culture media.

4.1.3.9) Fluorescence analysis

The powder of crude plant materials of *Glycyrrhiza glabra* and *Nelumbo nucifera* were treated with different reagents.

4.1.3.10) Qualitative Phytochemical Screening

The petroleum ether, toluene, chloroform, ethyl acetate, acetone, methanol and water were studied to determine the chemical constituents in the plants.

4.1.3.10.1) Extraction

The above mentioned solvents are good for the preliminary extraction and most classes of compounds show solubility in them. Hence the coarse powder of shade-dried plant was extracted. The extracts were concentrated and air-dried.

4.1.3.10.2) Chemical Tests [3]

The extracts were subjected to chemical tests for presence of following phytochemical classes like Carbohydrates, Alkaloids, Anthraquinones and Saponin glycosides, Phytosterols, Phenolics, Tannins, Flavonoids, Proteins and amino acids using reported methods.

4.1.3.10.3) Thin Layer Chromatographic studies [5]

The extracts obtained were subjected to thin layer chromatographic studies using reported methods to determine the presence of various phytoconstituents. The results were compared with the results obtained in the qualitative tests. The mobile phases and detecting reagents of various classes of compounds are mentioned in Table 4.1.

Table 4.1: Detection conditions of plant constituents using TLC

Class of Compound	Solvent system	Detection	Identification
Anthraglycosides	Ethyl acetate: methanol: water (100:13.5:10)	KOH Reagent	Red: Anthraquinone Yellow: Anthrones
Alkaloids	Ethyl acetate: methanol: water (100:13.5:10)	Dragendorff's Reagent	Orange Brown
Arbutin like Compounds	Ethyl acetate: methanol: water	Berlin Blue	Blue

	(100:13.5:10)		
Bitter Principles	Ethyl acetate: methanol: water (100:13.5:10)	Vanillin – Sulphuric Acid	Red/Yellow-Brown/Blue-green
Saponins	Chloroform: glacial acetic acid: methanol: water (64: 32: 12:8)	A.S. Reagent	Blue
Essential Oils	Toluene: Ethyl acetate (93:7)	V.S. Reagent	Red/Yellow/Blue/ Brown (visible)
Flavonoids	Ethyl acetate: Formic acid: glacial acetic acid: water (100:11: 11:26)	$AlCl_3$ reagent/ NP-PEG regent	Yellow/green/orange (UV-365nm)

4.1.3.11) Estimation of secondary metabolites

4.1.3.11.1) Determination of total phenolic content

The phenolic content in the water extract of *Glycyrrhiza glabra, Prunella vulgaris, Zizyphus jujube* and hydro alcoholic extract of *Nelumbo nucifera* were determined as per the reported method.

Preparation of test sample

To dissolve 10mg of each extract in 10ml methanol to get the stock solutions of samples (1000 µg/ml).

Preparation of Reagent

Folin ciocalteu reagent: 1:2 dilution of the reagent with distilled water was prepared.

20% sodium carbonate solution: 20gms of anhydrous sodium carbonate was dissolved in 100ml of distilled water.

Protocol for the total phenolic content

1. In 25ml of volumetric flask, 1ml of sample was taken. Then 10ml of water and 1.5ml of Folin Ciocalteu reagent was added. The above mixture was kept for 5

min at room temperarture and then 4 ml of 20% sodium carbonate (Na_2CO_3) solution was added. Finally the volume was made up to 25ml with distilled water. This mixture was kept for 30min at room temperature. The blue colour was developed in the solutions. The absorbance of this was measured at 765nm.

2. From the stock solution of standard 0.5, 0.75, 1.0, 1.25, 1.75 and 2ml were taken which gave 50, 75, 100, 125, 150, 175 and 200µg/ml concentrations respectively. Percentage of total phenolics was calculated from the calibration curve of gallic acid plotted by using the above procedure and total phenolics were expressed as % gallic acid.

4.1.3.11.2) Estimation of total flavonoid content

The total flavonoid content was determined by two methods and the sum of their giving the total flavonoids present in them. In both the method the flavonoid content was measured in the water extracts of *Glycyrrhiza glabra, Prunella vulgaris, Zizyphus jujube* and hydro alcoholic extract of *Nelumbo nucifera.* The sample solutions of 1.5mg/ml and 5mg/ml were prepared for estimation of total flavonoid content by Aluminium chloride colorimetric method and 2, 4- dinitrophenyl hydrazine colorimetric method respectively.

4.1.3.11.2.1) Aluminium Chloride Colorimetric Method

Preparation of standard solution

A stock solution of 1mg/10ml of quercetin was prepared in methanol.

Preparation of reagents

10% aluminium chloride: 10gms of aluminium chloride was dissolved in 100ml of distilled water.

1M potassium acetate: 9.814gms of potassium acetate were dissolved in 100ml distilled water.

Protocol for total flavonoid content

1. Standard quercetin solutions were used to make the calibration curve.
2. For the stock solution of standard 0.1, 0.2, 0.3, 0.4 and 0.5 ml were taken which gave 10, 20, 30, 40 and 50 µg/ml concentrations respectively.
3. The standard solution were separately mixed with 1.5ml of methanol (MeOH), 0.1ml of 10% aluminium chloride ($AlCl_3$), 0.1ml of 1 M potassium acetate and 2.8ml of distilled water.

4. The mixture was incubated at room temperature for 30 min and then the absorbance of reaction mixture was measured at 415nm.
5. The volume of 10% aluminum chloride solution was substituted by the same volume of distilled water in blank solution.
6. Similarly, 1.5mg/ml (1ml) of the sample solution were reacted with aluminum chloride for determination of flavonoid content as described in above procedure.

4.1.3.11.2.2) 2, 4-Dinitrophenylhydrazine Colorimetric Method

Preparation of Standard solution

A stock solution of 10mg/10ml of Naringin was prepared in distilled water.

Preparation of reagent solution

1% 2, 4-dinitrophenyl hydrazine (2,4-DNPH) reagent: 1 gm 2,4-DNPH was dissolved in 2ml 96% sulphuric acid (H_2SO_4) and then diluted up to 100 ml with distilled water.

1% Potassium hydroxide: 1 gm of potassium hydroxide was dissolved in 100ml of 70% methanol.

Protocol for total flavonoid content

1. Standard solutions of naringin were used to make the calibration curve.
2. Required 1 ml of standard stock solution were taken and diluted to give 250, 500, 1000, 1500, 2000 µg/ml concentrations respectively.
3. 1ml of each standard solutions were separately mixed with 2ml of 1% 2, 4-DNPH reagent and 2ml of methanol and then kept at 50 °C for 50min.
4. After cooling to room temperature, the reaction mixtures were mixed with 5ml of 1% potassium hydroxide in 70 % methanol and incubated at room temperature for 2 min.
5. Then, 1ml of the mixture was taken, mixed with 5ml of methanol and centrifuged at 100 rpm for 10 min to remove the precipitates.
6. The supernatant was collected and adjusted to 25 m. The absorbance of the supernatant was measured at 495nm.
7. For determination of flavonoid content, similarly, 5mg/ml sample solution were reacted with 2, 4- DNPH as described in the above procedure.

4.1.4) *In- vitro* antioxidant activity

The root extract of *G. Glabra*, leaf extract of *N. nucifera*, fruit extract of *Z. jujuba* and aerial parts' extract *of P. vulgaris* were evaluated for their antioxidant property.

4.1.4.1) DPPH Free radical scavenging activity

Preparation of standard solution

The sufficient amount of ascorbic acid was dissolved in methanol to give the concentration of 10, 15, 20, 25, 30 and 35µg/ml.

Preparation of sample stock solution

The individual test samples were dissolved in methanol to give stock solutions of 10mg/ml.

Preparation of test samples

25, 50, 100, 200, 300, 400µg/ml concentration of the test samples were prepared by proper dilution of the stock solution with methanol.

Preparation of (1, 1 diphenyl 2- picryl hydrazine) DPPH solution

1.3mg of DPPH was dissolved in 1ml of methanol. It was protected from the light by using amber coloured volumetric flask and also with aluminium foil.

Protocol for estimation of DPPH free radical scavenging activity

1. 75µl DPPH solution was added to 3ml methanol and the absorbance was taken immediately at 516nm for control reading.
2. Different dose levels were screened i.e. 25-400 µg/ml. For this 1 ml from each test samples were added with 3ml methanol in amber volumetric flask.
3. 75 µl DPPH was added to each vial.
4. The absorbance was taken immediately after condition of DPPH solution at 516nm using methanol as a blank at zero minute.
5. Decrease in absorbance in presence of test samples at different concentration was noted after 30min.

The percentage inhibition of DPPH radical was calculated by comparing the results of the test with those of the control (without extract) using the formula:

$$\text{Percentage inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of test})}{\text{Absorbance of control}} \times 100$$

4.1.4.2) Antioxidant capacity by Phosphomolybdanum complex method

Preparation of Standard solution

Stock solution of ascorbic acid (100µg/ml) was prepared in methanol. Aliquots of 0.1, 0.5, 1.0, 1.5, 2.0 and 2.5ml were taken from stock solution and diluted up to 10ml with methanol to get the concentrations of 1, 5, 10, 15, 20 and 25µg/ml respectively.

Preparation of sample solution

The individual test samples were dissolved in sufficient quantity of methanol to give stock solution of 1mg/ml. The stock solutions were further diluted with phosphate buffer to give the concentration of 50,100,150 and 200 µg/ml.

Preparation of reagent solution

0.6M sulphuric acid: 3.24ml concentrated sulphuric acid is dissolved in 100ml of distilled water. 4mM ammonium molybdate: 0.49g of ammonium molybdate is dissolved in 100ml of distilled water.

Procedure

An aliquot of 0.1ml of each sample and standard solutions in methanol were combined in an eppendorf tube with 1ml of reagent solution. The tubes were capped and incubated in a water bath at 95ºC for 90min. The absorbance of each solution was measured at 695nm against a blank solution using UV spectrophotometer when the samples were cooled at room temperature. A typical blank solution contained 1ml of reagent solution and the appropriate volume of the same solvent used for the samples and it was incubated under the same conditions as the rest of the samples. The samples were as equivalents of ascorbic acid.

4.2) RESULTS AND DISCUSSIONS

4.2.1) Plant material

Dried roots of *Glycyrrhiza glabra* and *leaves of Nelumbo nucifera* were authentified by Prof. P. S. Nagar, Botany Department, The M. S. University of Baroda, Vadodara. The herbarium specimens (No. 2A, 3A), (No.2B, 3B), Ref: Bot/20214/aut respectively was submitted in the Pharmacy Department, The M.S. University of Baroda, Vadodara.

4.2.2) Standardization of the plant materials as per the WHO guidelines

4.2.2.1) Macroscopic evaluation

All the organoleptic features of *Glycyrrhiza glabra* (WHO monographs) and *Nelumbonucifera* (P. mukherji) were found to match with that of the reported monographs.

(a) Glycyrrhiza glabra

(b) Dried roots of *Glycyrrhiza glabra*

Fig. 4.1 (a) Photograph of *Glycyrrhiza glabra* (b) Dried roots of *Glycyrrhiza glabra*

(a) Fresh leaves of *Nelumbo nucifera*

(b) Dried leaves of *Nelumbo nucifera*

Fig. 4.2 (a) Fresh leaves of *Nelumbo nucifera (b)* Dried leaves of *Nelumbo nucifera*

4.2.2.2) Microscopical and Histological analysis

Detailed anatomical features of the roots were studied. Transverse section of *Glycyrrhiza glabra* root is circular in outline and shows the following regions (Fig.4.3).

Cork: Radially arranged, thin walled, polygonal, tabular cells.

Pheloderm: It is present below the cork

Cortex: It is made up of parenchymatous cells.

Pericycle: Small groups of pericyclic fibres at intervals.

Phloem: It consists of fibres, slightly lignified alternating with sieve tissue.

Ceratenchyma: Collapsed sieve tissue on the outer side of the phloem.

Xylem: It is made up of xylem fibres, vessels and little xylem parenchyma. Four small primary xylem bundles, arranged at right angles to each other, protoxylem is directing outward.

Medullary rays: Cellulosic parenchyma radially elongated cells.

Calcium oxalate crystals: It is present in parenchymatous cells.

Starch grains: It is present in the rest of paenchymatous cells, round in shape.

Pith: It is made up of parenchymatous cells.

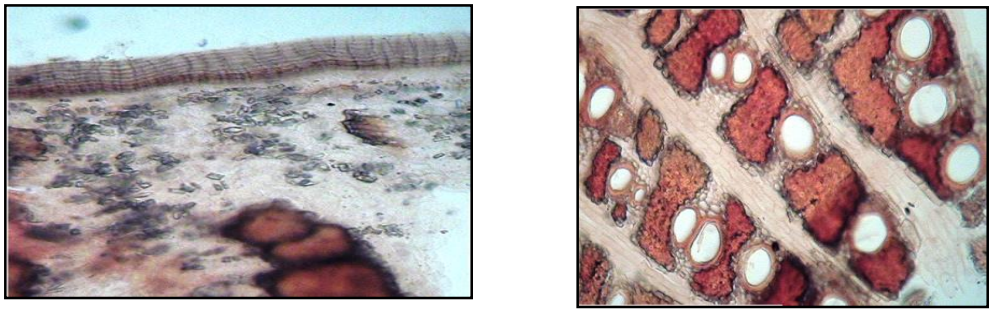
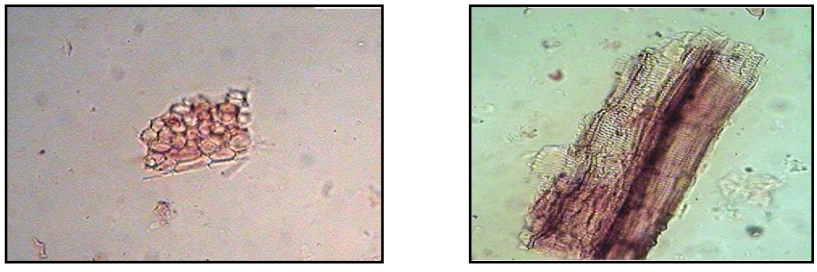


Fig. 4.3 Transverse sections of *Glycyrrhiza glabra* roots

The powdered drug also showed the presence of similar microscopical structures as shown in Fig.4.4.



(a) (b)

(c) (d)

Fi.g 4.4 Diagnostic features for the powder microscopy of the roots of *Glycyrrhiza glabra* (a) Cork cells (b) Pitted xylem vessels (c) Bundles of xylem Fibres (d) Calcium oxalate crystals

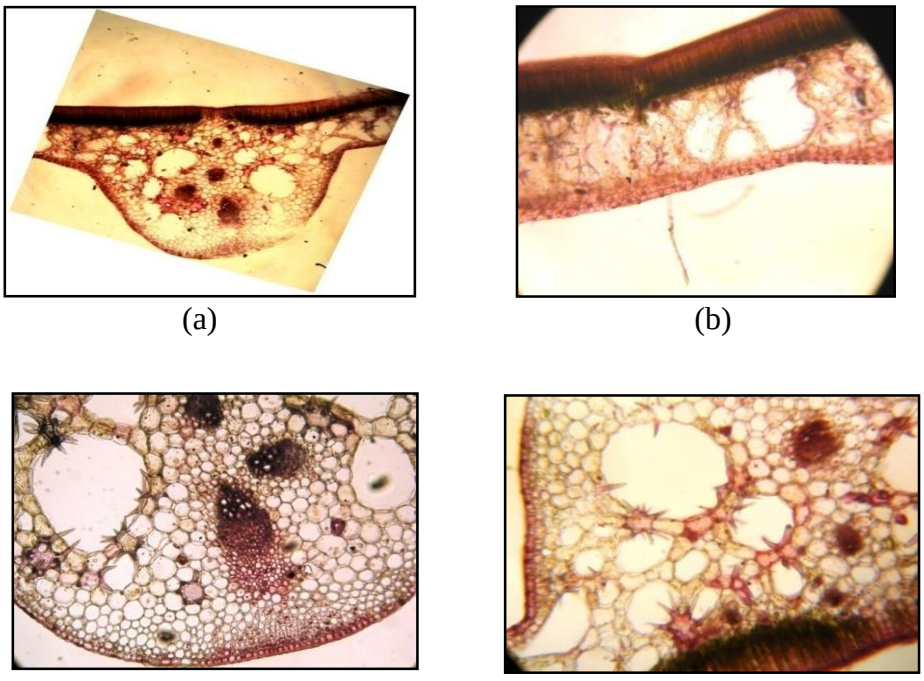
Transverse section of *Nelumbo nucifera* leaf shows the following regions (Fig.4.5).

Lower and upper epidermis: It consists of thin walled polygonal cells with wavy margin. Lower epidermis shows abundant anomocytic stomata and branch multiseriate trichomes.

Mesophyll: It is made up of single layer palisade tissue.

Midrib: It shows scattered vascular bundles containing xylem and phloem. It also shows large cavities in the center and small cavities in the periphery.

Collenchyma: A distinct collenchymatous layer is present above the lower epidermis.



(a) (b)

(c) (d)

Fig. 4.5 Transverse section of *Nelumbo nucifera leaf* (a) Mid rib (b) Lamina (c) Lower part of mid rib (d) Mesophyll showing cavities

The powdered drug also showed the presence of similar microscopical structures as shown in Fig.4.6.

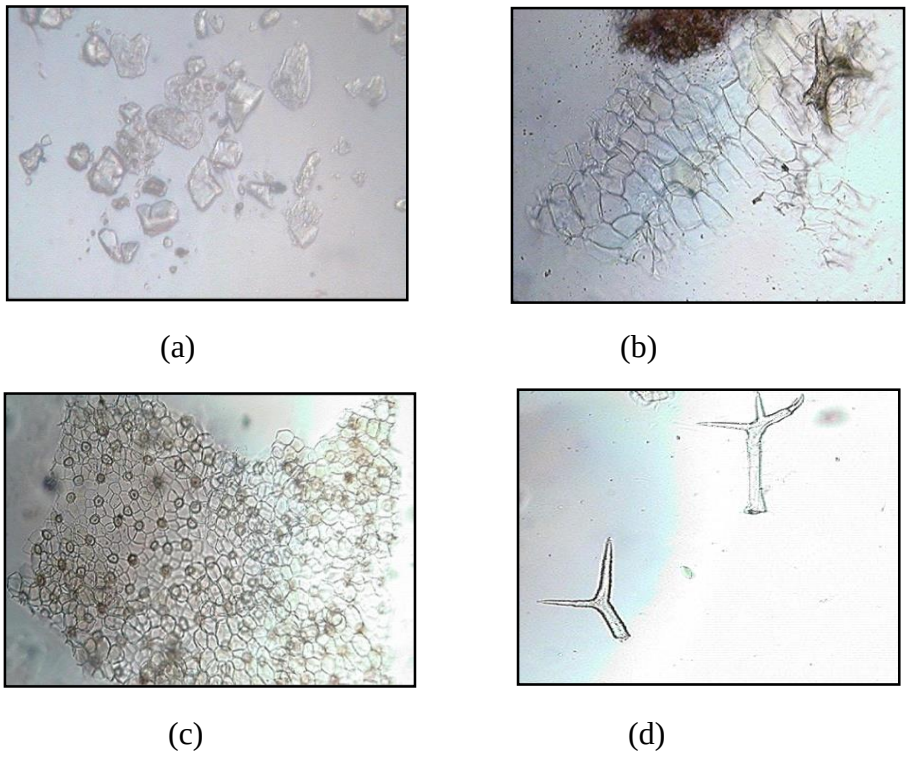


Fig 4.6 Diagnostic features for the powder microscopy of the leaves of *Nelumbo nucifera* (a) Calcium oxalate prisms (b) Lamina (c) Anomocytic stomata (d) Branch trichome

4.2.2.3) Determination of ash

The Table 4.2 shows the ash values of all the four plant materials. All the values of total ash comply with the prescribed limits in the WHO monographs. The water soluble components are higher in concentration as compared to the insoluble components.

Table 4.2 Ash values of plant materials (n=3)

Sample	Total ash (%)	Acid insoluble ash (%)	Water soluble ash (%)
G. glabra	7.5 ± 0.12	1.5 ± 0.05	1.5 ± 0.09
N. nucifera	4.26 ± 0.15	2.31 ± 0.09	0.96 ± 0.1

The inorganic content of the plant materials are determined by the ash value. The acid insoluble ash is the total soil or silicieous matter present in the plants. Thus ash value of the plant material determines the raw material quality of the plant materials. The results showed that the ash values are within the prescribed limits. (Herbal p'copiea)

4.2.2.4) Determination of Extractable matter

Coarse powder of the dried plant materials was extracted with petroleum ether, methanol and water, separately, in a soxhlet extractor; the extract was concentrated and dried under vacuum. The extractive values of the plant materials in the three solvents have been given in Table 4.3.

Table 4.3 Extractive values of plant materials (n=3)

Sample	Water soluble extractive value %	Alcohol soluble extractive value (%)	Ether soluble extractive value (%)
G. glabra	9.95 ± 0.14	13.13 ± 0.22	6.2 ± 0.19
N. nucifera	8.71 ± 0.18	7.84 ± 0.27	5.4 ± 0.2

In the absence of the known active constituents, the different classes of compounds extracted in different solvents become important standardization parameter. The extractive values in all the solvents of all the plant materials are in accordance with the WHO monographs. (Herbal p'copiea)

4.2.2.5) Determination of water and volatile matter

The % loss on drying of the plant materials is given in Table 4.4. The values comply with those of the limits prescribed in the standard monographs. (Herbal pharmacopeia)

Table 4.4 % Loss on drying of plant materials (n=3)

Sample	***% Loss on drying***
G. glabra	3.8 ± 0.07
N. nucifera	4.62 ± 0.33

The quality of the plant material has to be maintained for which the degradation conditions have to be minimised. The moisture content should therefore be

determined. In the present study, the % loss on drying of all the plant materials was found to be within the limits.

4.2.2.6) Determination of pesticides residue

The TLC studies were performed for detection of possible organochorinated pesticides and organophosphorus pesticides. The results of the analysis are shown in Table 4.5.

Table 4.5 Analysis for Pesticide residues in plant materials

Sample	**Organochlorinated Pesticides**		**Organophosphorus pesticides**	
	A	B	C	D
G.glabra	-ve	-ve	-ve	-ve
N. nucifera	-ve	-ve	-ve	-ve

-ve = Negative

A- Solvent system: DMF: Ether (4:6), Detecting Reagent: Tetrabromophenolphthalein.

B- Solvent system: Methylcyclohexane, Detecting Reagent: Tetrabromophenolphthalein.

C- Solvent system: DMF: Ether (4:6), Detecting Reagent: 0.5 % Silver nitrate in water and acetone.

D- Solvent system: DMF: Ether (4:6), Detecting Reagent: 0.5 % Silver nitrate in water and acetone.

The results of the tests for pesticide residues showed the absence of organochlorinated and organophosphorus pesticides. Pesticide residues are the important parameter for standardization of the plant materials as plant materials are generally prone to be contaminated with such substances and may prove to be harmful on long term usage.

4.2.2.7) Determination of Heavy metals

Elemental analysis of plant materials was done by Atomic absorption spectrophotometer, where Lead, Cadmium, Mercury and Arsenic were found to be absent. (Table.4.6)

Table 4.6 Heavy metal analysis of plant materials

Sample	*Cadmium (not more than 0.30 ppm)*	*Arsenic (not more than 10 ppm)*	*Mercury (not more than 1ppm)*	*Lead (not more than 10 ppm)*
G. glabra	0.203	1.14	absent	3.63
N. nucifera	0.287	0.76	absent	5.14

The determination of heavy metals in the plant material showed the absence of mercury and cadmium, arsenic and lead are within the limits. The determination of these heavy metals is important for standardization because if their content is above the prescribed limit, the heavy metals prove to be toxic on long term usage.

4.2.2.8) Determination of microbial contamination

The analysis of bio-burden present in the plant materials was performed and all the results were within the limits prescribed in the WHO guidelines (Table 4.7). The total microbial contamination in the plant materials should be determined for the quality control because these plant materials have to be taken internally and may prove to be harmful in a number of cases. The presence of microbial bioburden also leads to degradation of the active constituents which may lead to the loss of activity of the formulation.

Table 4.7 Results of the tests performed for the microbial contamination in the plant materials

Parameters	*G. glabra*	*N. nucifera*
Total bacterial count	> 10,000 cfu/gm	> 10,000 cfu/gm
E.coli	Absent	Absent
Salmonella. Typhii	Absent	Absent
S,aureus	Absent	Absent
Pseudomonas aeruginosa	Absent	Absent
Yeast and mould	Absent	Absent

4.2.2.9) Fluorescence analysis

The fluorescence analysis of powdered drug plays an important role in the determination of quality and purity of the plant material. In this study, powder treated

with various reagents shows characteristic fluorescence at 254 nm and 366 nm wavelength (Table 4.8 and 4.9).

Table 4.8: Fluorescence analysis of *Glycyrrhiza glabra* powder under day light and ultra violet (UV) radiation

Sr.No.	Treatment	Fluorescence	
		Day light	UV light
1.	Powder as such	Light brown	Bright brown
2.	Powder + 1N NaOH (Aq.)	Dark Brown	brownish black
3.	Powder+ 1 N NaOH (MeOH)	Light brown	Dark brown
4.	Powder+ 1 N HCl (Aq.)	Reddish yellow	Brown
5.	Powder + Iodine (N/50)	Dark brown	Black
6.	Powder + 50% H_2SO_4	Brownish orange	Black
7.	Powder + 50 % HNO_3	Reddish yellow	Dark brown

Table 4.9: Fluorescence analysis of *Nelumbo nucifera* leaf powder under day light and ultra violet (UV) radiation

Sr.No.	Treatment	Fluorescence	
		Day light	UV light
1.	Powder as such	Light green	Bright green
2.	Powder + 1N NaOH (Aq.)	Brown	Greenish black
3.	Powder+ 1 N NaOH (MeOH)	Light green	Dark green
4.	Powder+ 1 N HCl (Aq.)	Reddish yellow	Green
5.	Powder + Iodine (N/50)	Dark green	Black
6.	Powder + 50% H_2SO_4	Brownish orange	Black
7.	Powder + 50 % HNO_3	Reddish yellow	Dark green

4.2.2.10) Qualitative Phytochemical Screening

The phytochemical screening of the plant materials was done by qualitative chemical tests and TLC studies. Qualitative chemical tests were performed for petroleum ether extract, toluene, choloform, ethyl acetate, acetone, methanol and the aqueous extract. The preliminary phytochemical studies of *Glycyrhhiza glabra* revealed that petroleum ether fraction contain fixed oil and fats, toluene fraction also contains fixed oils, chloroform fraction contain phenolics, acetone fraction contains carbohydrate, phytosterol, saponin and flavanoids and phenolics, while methanolic fraction contain carbohydrate, phytosterol, saponin, proteins and amino acid, phenolic and flavonoids. The percentage yield values for petroleum ether, toluene, chloroform, ethyl acetate, acetone and methanol were 4.42%, 3.17%, 2.44%, 2.19%, 5.71% and 8.36% respectively (Table 4.10). All the values were found to be within the limits.

Table: 4.10 Preliminary phytoprofile of *Glycyrrhiza glabra* root.

Extract	Colour & consistency after drying	Average value of extract in %	Constituents							
			A	C	P	F	S	Pr	Fl	Ph
P. Ether (60-80°c)	Light brown, semi solid	1.61%	-	-	+	-	-	-	-	-
Toluene	Reddish brown, semi solid	3.73%	-	-	-	-	-	-	-	-
Chloroform	Brownish, solid	1.42%	-	-	+	-	-	-	-	-
Ethyl acetate	Brown, solid	3.26%	-	-	+	-	-	-	-	-
Acetone	Brown, semi solid	1.58%	-	+	+	-	+	-	+	-
Methanol	Brown, solid	13.13%	-	+	+	-	+	+	+	+
Water	Dark brown, solid	9.95%	-	+	-	-	+	+	-	+

(A-Alkaloids, S-Saponins, C -Carbohydrates and Glycosides, Pr- Proteins & Amino acids, P- Phytosterols, Fl-Flavonoid, F-Fixed Oils, Fats & Waxes, Ph-Phenolic)

- : Negative, + : Positive

The preliminary phytochemical studies of *Nelumbo nucifera* revealed that petroleum ether fraction contain fixed oil and fats, toluene fraction also contains fixed oils, chloroform fraction contain carbohydrate and saponins, acetone fraction contains carbohydrate, saponin and flavanoids and phenolics, while methanolic fraction contain carbohydrate, proteins and amino acids, saponin, phenolic and flavonoids. The percentage yield values for petroleum ether, toluene, chloroform, ethyl acetate, acetone, methanol and water were 1.61%, 3.73%, 1.42%, 3.26%, 1.58%, 13.13% and 9.95% respectively (Table 5.10). All the values were found to be within the limits.

Table: 4.11 Preliminary phytoprofile of *Nelumbo nucifera* leaves

Extract	Colour & consistency after drying	Average value of extract in %	Constituents							
			A	C	P	F	S	Pr	Fl	Ph
P. Ether (60-80°c)	Green, solid	4.42%	-	-	+	-	-	-	-	-
Toluene	Dark green, solid	3.17%	-	-	-	-	-	-	-	-
Chloroform	Blackish green residue	2.44%	+	-	-	-	-	-	-	-
Ethyl acetate	Brown, solid	2.19%	-	-	+	-	-	-	-	-
Acetone	Dark brown semi solid	5.71%	-	-	+	-	+	-	+	-
Methanol	Reddish Brown, semi solid	8.36%	+	+	+	-	-	+	+	-
Water	Dark green, semi solid	9.95%	+	+	-	-	-	+	-	-

TLC studies were performed for the crude drug extracts of *Glycyrrhiza glabra* and *Nelumbo nucifera.*

Table 4.12 TLC Screening of various crude drug extract of *Glycyrrhiza glabra* roots

Solvent system Used	Detection Reagent	Observation	Inference	P	T	C	A	M
Toluene: Ethyl acetate	VS reagent	Red/Yellow/Brown/ Blue-green	Essential Oil	+	+	-	-	-

(93:7)	AS reagent	Pink/green	Essential Oil	+	+	-	-	-
Ethyl acetate : Methanol : Water : (100:13.5:10)	AS reagent	Red/Yellow/Brown/ Blue-green	Bitter Principle	+	+	-	-	-
	VS reagent	Blue	Saponin	-	-	+	+	+
	NP/PEG/ and UV	Yellow colour	Flavonoid	-	-	-	+	+
	10% $AlCl_3$	Yellow colour	Anthrone	-	+	+	+	+
Chloroform : Methanol : Formic acid (10:0.3:0.1)	LB reagent	Dark green	Phytosterol	+	-	-	-	-

Table 4.13 TLC Screening of various crude drug extract of *Nelumbo nucifera* leaves

Solvent system Used	**Detection Reagent**	**Observation**	**Inference**	**P**	**T**	**C**	**A**	**M**
Toluene: Ethyl acetate (93:7)	VS reagent	Red/Yellow/Brown/ Blue-green	Essential Oil	+	+	-	-	-
	AS reagent	Pink/green	Essential Oil	+	+	-	-	-
Ethyl acetate : Methanol : Water : (100:13.5:10)	AS reagent	Red/Yellow/Brown/ Blue-green	Bitter Principle	+	+	-	-	-
	VS reagent	Blue	Saponin	-	-	-	-	-
	NP/PEG/ and UV	Yellow colour	Flavonoid	-	-	-	+	+

	10% $AlCl_3$	Yellow colour	Anthrone	-	+	+	+	+
Chloroform : Methanol : Formic acid (10:0.3:0.1)	LB reagent	Dark green	Phytosterol	+	-	-	-	-

(P= Petroleum ether extract; T= Toluene extract; C= Chloroform extract; A= Acetone extract; M= Methanol extract, VS = Vanillin sulphuric acid, AS = Anisaldehyde sulphuric acid, LB = Libermann Burchard, + Indicates presence of constituents. – Indicates absence of constituents)

4.2.3) Estimation of secondary metabolites

Due to their phenolic and flavonoid content, complementary and alternative medicines containing radical scavengers exhibit efficient antioxidant property. The result of preliminary phytochemical analysis showed the presence of flavonoids and other phenolic constituents in the different extracts. Therefore the total flavonoids and phenolic contents were quantified in the water extracts of *G. glabra, P. vulgaris and Z. jujuba* and hydro alcoholic extracts of *N. nucifera.*

4.2.3.1) Determination of total phenolic content

Phenolic compound like gallic acid reacts with Folin-Ciocalteu reagent to form blue coloured complex. The estimation of phenolics involves the measurement of the absorbance of this coloured complex at 765nm. The total phenolic content was expressed in terms of % gallic acid. Phenolic content was determined in the extracts by above method and absorbance data is depicted in Table 4.14.

Total phenolic content was expressed as mg/g gallic acid equivalent using the following equation based on the calibration curve: $Y = 0.0042X + 0.0197$, $R^2 = 0.991$, where X was the absorbance and Y was the gallic acid equivalent (mg/g).

Table 4.14 Absorbance data of total phenolic content

Sample	Conc. (µg/ml)	Absorbance (nm)
Gallic acid	50	0.208
	75	0.336
	100	0.434
	125	0.579
	150	0.663
	175	0.752
	200	0.832
G. glabra	1 mg/ml	0.431
N. nucifera	0.5 mg/ml	0.757
P. vulgaris	1 mg/ml	0.344
Z. jujube	1 mg/ml	0.302

Table 4.15 Determination of total phenolic content of extracts

Extracts	% w/w Phenolic content
G. glabra	9.70 ± 0.6%
N. nucifera	35.11 ± 0.02 %
P. vulgaris	7.72 ± 0.34%
Z. jujuba	6.72 ± 0.08%

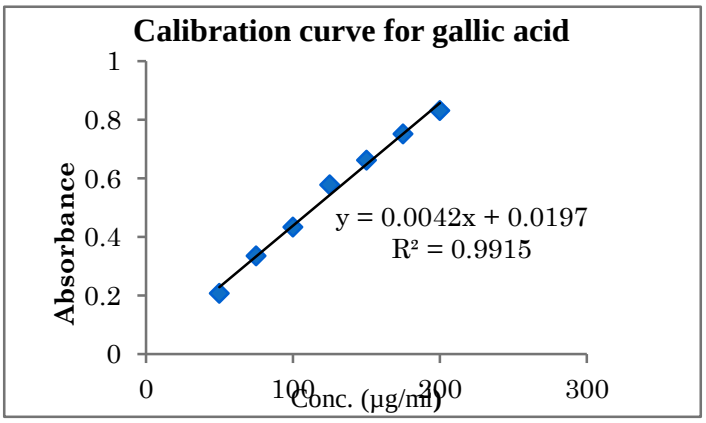


Fig. 4.7 Calibration curve of gallic acid

Total phenolic content in the water extract of *G. Glabra*, hydro alcoholic extract of *N. Nucifera, water extracts of P. vulgaris and Z. jujuba* were 9.70 ± 0.6%, 35.11±0.02% , 7.72 ± 0.34 and 6.72 ± 0.08% respectively (Table 4.15).

4.2.3.2) Determination of total flavonoid content

The total flavonoid content was determined by the two methods and the values obtained from these methods were added to give the final value of flavonoid contents. Flavones, flavonols and iso-flavones are formed complex mainly with Aluminum chloride ($AlCl_3$) while flavonones are strongly reacted only with 2, 4 – dinitrophenyl hydrazine (2, 4 DNPH) reagent. The results obtained by two methods were added up to evaluate the total flavonoid content.

4.2.3.2.1) Aluminum Chloride Colorimetric Method

The principle of $AlCl_3$ method is that it forms acid stable complexes with the flavones and flavonols. Quercetin is used as standard which is a flavonol reacts with $AlCl_3$ to form a stable complex. The calibration curve was plotted against concentration Vs absorbance. Y = 0.0138X - 0.0105, R^2 = 0.9916, where X was the absorbance and Y was the quercetin equivalent (mg/g). The co-efficient of determination (R^2) obtained was 0.9916.

4.2.3.2.2) 2, 4-Dinitrophenylhydrazine Colorimetric Method

The principle of 2, 4- DNPH method is that it reacts with ketones and aldehydes to form 2, 4 – dinitrophenylhydrazines which exhibit absorption maxima at 495 nm. Naringinin is used as standard which is a flavanone. The calibration curve was plotted against concentration Vs absorbance. The co-efficient of determination (R^2) obtained was 0.9919.

Total flavonoid content was calculated as quercetin and naringinin (mg/g) using the following equation based on the calibration curve: and Y= 5E-05X - 0.0074, R^2 = 0.9919, where X was the absorbance and Y was the naringinin equivalent (mg/g).

The total flavonoid content determined in the samples by Aluminum chloride method was found to be lower than that determined by 2, 4 – dinitrophenylhydrazine method.

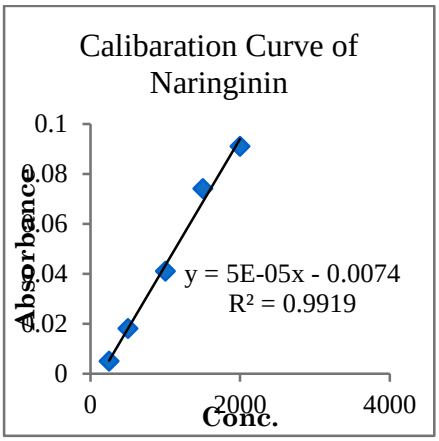
Table 4.16 Absorbance data of total flavonoid content (a) by aluminium chloride colorimetric method *(b)* by 2, 4-dinitrophenylhydrazine colorimetric method

Sample	Conc. (µg/ml)	Absorbance (nm)
Naringinin	250	0.005
	500	0.018
	1000	0.041
	1500	0.074
	2000	0.091
G.glabra	5.0 mg/ml	0.005
N.nucifera	5.0 mg/ml	0.065
P.vulgaris	5.0 mg/ml	0.017
Z.jujube	5.0 mg/ml	0.006

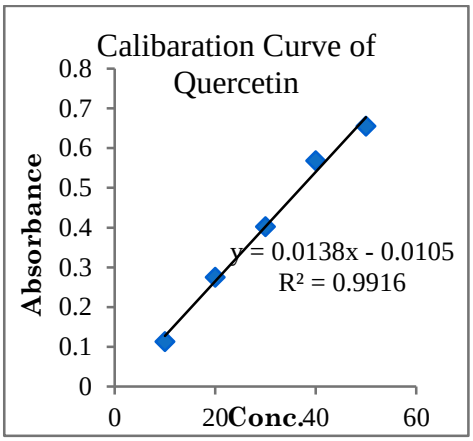
(a)

Sample	Conc. (µg/ml)	Absorbance (nm)
Quercetin	10	0.113
	20	0.275
	30	0.402
	40	0.568
	50	0.655
G.glabra	1.0 mg/ml	0.465
N.nucifera	1.0 mg/ml	0.488
P.vulgaris	1.0 mg/ml	0.271
Z.jujube	1.0 mg/ml	0.191

(b)



(a)



(b)

Fig. 4.8 (a) Calibaration Curve of Naringinin (b) Calibaration Curve of Quercetin

Table 4.17: Total flavonoid and phenolic content of plant extracts

Sample	Flavonoid content (%w/w)*			Total phenolic content (%w/w)*
	By $AlCl_3$ method	By 2,4 DNPH method	Total Flavonoid content	
Glycyrrhiza glabra	3.44 ± 0.06	4.88 ± 0.11	8.32 ± 0.17	11.7 ± 0.06

Nelumbo nucifera	3.61 ± 0.05	28.60 ± 0.03	32.21 ± 0.08	35.11 ± 0.02
Prunella vulgaris	2.04 ± 0.14	9.63 ± 0.06	11.67 ± 0.20	7.72 ± 0.12
Zizyphus jujube	1.46 ± 0.07	5.28 ± 0.08	6.74 ± 0.15	6.72 ± 0.04

*Results were presented as mean ± SD (n=3).

4.2.4) Preliminary *in- vitro* antioxidant activity

The preliminary *in-vitro* antioxidant activity was done by two methods such as DPPH free radical scavenging activity and phosphomolybdanum complex method.

4.2.4.1) DPPH Free radical scavenging activity

This activity was expressed as decrease in absorbance of the samples at a different concentration levels. The results are tabulated in Table 4.18 and are expressed in the form of graph. EC_{50} was calculated by the Graph pad prism 5. i. e. the concentration of the sample required to give the 50 % decrease in the absorbance compared to that of control reading.

Table 4.18 DPPH Free radical scavenging activity

Standard data		**Samples data(% Reduction)**				
Ascorbic acid (Asc)			***G. glabra (A)***	***N. nucifera (B)***	***P. vulgaris (C)***	***Z. jujuba (D)***
Conc. (µg/ml)	**% Red.**	**Conc. (µg/ml)**				
10	33.02	25	36.47	37.01	40.25	30.80
20	47.24	50	44.78	49.56	42.35	40.11
40	63.05	100	54.85	54.60	49.74	49.53
60	76.34	200	65.57	55.83	64.34	64.44
80	87.3	300	80.77	58.96	81.73	79.64
100	90.2	400	91.48	65.39	82.46	86.78
EC_{50}	**26.17**		**55.82**	**50.44**	**100.52**	**62.32**

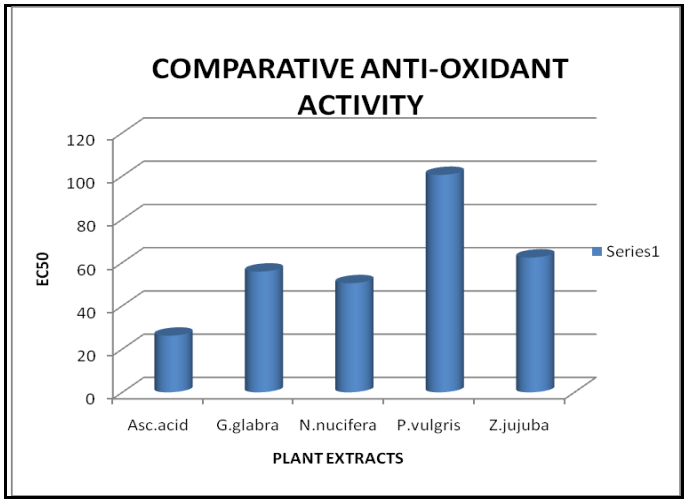


Fig. 4.9 Comparative DPPH free radical scavenging activity

The EC_{50} values of water extract of *G. Glabra*, hydro alcoholic extract of *N. Nucifera*, water extracts of *P. vulgaris and Z. jujuba* were 55.82, 50.44, 100.52 and 62.32 % obtained by DPPH method.

4.2.4.2) Antioxidant capacity by phosphomolybdanum complex

All the four extracts were analysed for antioxidant capacity by phosphomolybdanum complex method. It is a spectroscopic method for the quantitative determination of antioxidant capacity, through the formation of phosphor molybdenum complex. The assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and subsequent formation of a green phosphate Mo (V) complex which is measured at 695 nm.

Total antioxidant capacity of the samples was expressed as the number of gram equivalents of ascorbic acid. (Table 4.19) Again, *Nelumbo nucifera* extract showed better antioxidant capacity compared to all extracts.

Table 4.19 Absorbance data of ascorbic acid

Concentration (µg/ml)	Absorbance (nm)
5	0.042
10	0.062
20	0.102
30	0.14
40	0.177
50	0.216

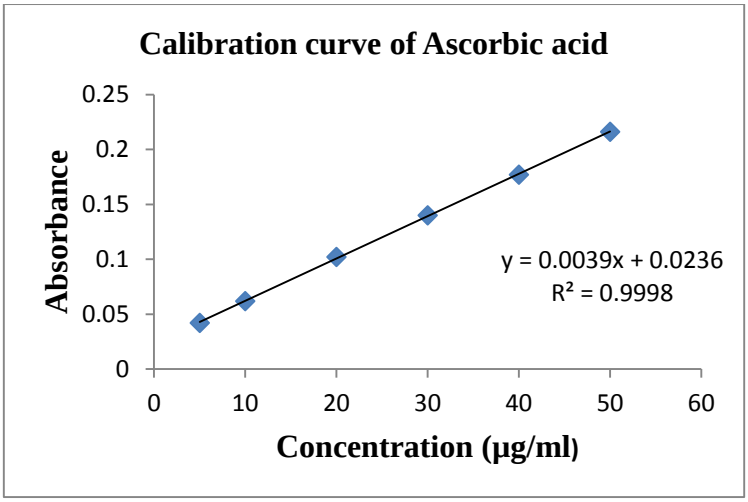


Fig. 4.10 Calibration curve of ascorbic acid

Table 4.20 Concentration of samples equivalent to ascorbic acid by Phosphomolybdanum complex method

Concentration of test samples (µg/ml)	Concentration equivalent to ascorbic acid			
	G. glabra	*N. nucifera*	*P. Vulgaris*	*Z. jujuba*
20	-4.1	-0.75	-7.88	-11.25
40	-0.284	11.2	-4.64	-6.51
60	4.3	21.43	-1.97	-1.44
80	8.1	28.22	3.55	3.82
100	12.26	39	5.7	6.66

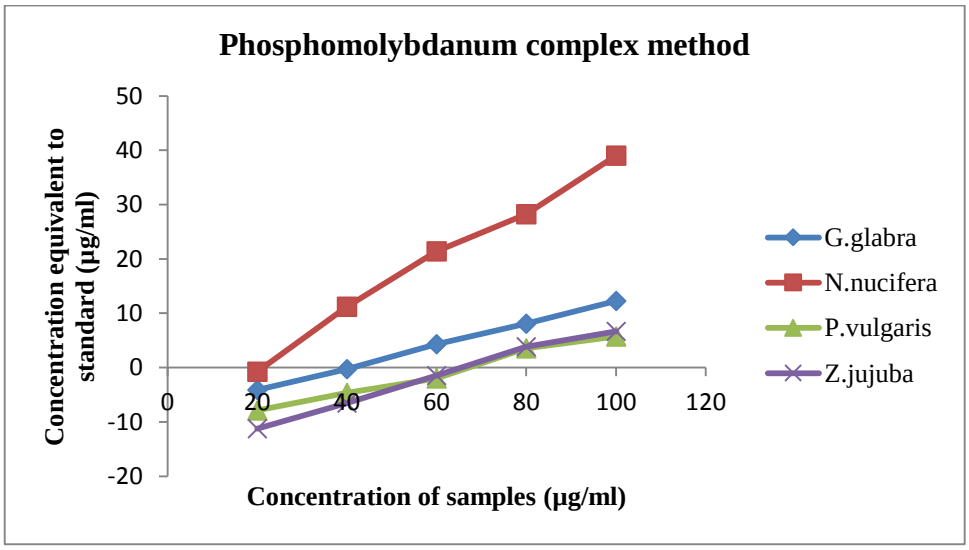


Fig. 4.11 Comparative phosphomolybdanum complex method

4.3) CONCLUSION

The plant materials were studied as per the WHO guidelines. The macroscopical and microscopical examination of the crude intact drugs (*G. glabra* roots and *N. nucifera* leaves) and powdered drugs was done. The determination of ash values, extractive values, moisture content, volatile matter, pesticide residues, heavy metals, microbial content was also performed. All these parameters showed that the plant material complied with the limits prescribed in the WHO guidelines. The physically standardized plant materials were extracted in petroleum ether, toluene, chloroform, ethyl acetate, acetone, methanol and the aqueous extract. These extracts were then subjected to preliminary phytochemical analysis using chemical tests and TLC studies which revealed the presence of saponins, carbohydrates and glycosides, proteins and amino acids, phytosterols, flavonoid and phenolics in *G. glabra* root extract. The *N. nucifera* leaves extract revealed the presence of alkaloids, carbohydrates and glycosides, proteins and amino acids, phytosterols and flavonoids.

Complementary and alternative medicines containing radical scavengers exhibit efficient antioxidant activity due to their phenolic and flavonoid content. The result of preliminary phytochemical analysis showed the presence of flavonoids and other phenolic constituents in the different extracts. Therefore the total flavonoids and phenolic contents were quantified in the water extracts of *G. glabra, P. vulgaris and Z. jujuba* and hydro alcoholic extracts of *N. nucifera.* Total phenolic content in the water extract of *G. Glabra*, hydro alcoholic extract of *N. Nucifera, aqueous extracts of P. vulgaris and Z. jujuba* were 9.70 ± 0.6%, 35.11±0.02% , 7.72 ± 0.34 and 6.72 ± 0.08% respectively. Total flavonoid content in the water extract of *G. Glabra*, hydro alcoholic extract of *N. Nucifera,* aqueous extracts of *P. vulgaris* and *Z. jujuba* were 8.32 ± 0.17%, 32.21 ± 0.08%, 11.67 ± 0.20%, 6.74 ± 0.15% respectively.

The preliminary *in-vitro* antioxidant activity was done by two methods, DPPH free radical scavenging activity and phosphomolybdanum complex method. The EC_{50} values of water extract of *G. Glabra*, hydro alcoholic extract of *N. Nucifera,* water extracts of *P. vulgaris and Z. jujuba* were 55.82, 50.44, 100.52 and 62.32 % obtained by DPPH method. The values suggest that *N. nucifera* has highest antioxidant activity compare to other three extracts. Total antioxidant capacity of the extracts was expressed as the number of gram equivalents of ascorbic acid by phosphomolybdanum complex method. *Nelumbo nucifera* extract showed better antioxidant capacity when compared to other extracts.

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