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Hypolipidemic Activity of Standardized Crude Leaf Extracts of *Morus nigra* L.

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Abstract:

Morus nigra belongs to moraceae family. Present study focused on hypolipidemic activity, macroscopic, microscopic characters as well as physicochemical parameters of the leaf of *Morus nigra*. Results show the hypolipidemic activity along with standardization of crude drugs. Physicochemical and fluorescence characteristics have also been investigated. Morphologically leaves of *Morus nigra* are 7 to 12 cm in length, alternate, serrate and broadly ovate. Microscopy of leaf shows various uniseriate covering trichomes, thick cuticle and some glandular trichomes, stomata are anomocytic type, palisade cells, spongy parenchyma, collenchyma, rosette calcium oxalate crystals, starch granules and radially arranged vascular bundles. Some parameters such as moisture content, extractive value, ash value, foreign organic matter, and foaming index were analyzed. Leaf constants such as stomatal number, stomatal index, vein islet number, vein termination number and palisade ratio also determined. The hypolipidemic activity was carried out in male SD rats. Triton X-100 was used for the induction of hyperlipidemia in rats by intraperitoneal administration. 300 mg /kg body weight dose of hydroalcoholic extract of test drug given orally and 10mg /kg body weight dose of atorvastatin drug used as standard compound. Test drug shows reduction in total cholesterol, triglycerides, low-density lipoprotein, while high-density lipoprotein has been increased.

Keywords: *Microscopic, Physicochemical, Organoleptic, Fluorescence Analysis, Hypolipidemic*

INTRODUCTION

Medicines are the medium for saving the lives of human beings from different diseases. These medicines are manufactured on the lab-scale or the industrial scale, and it can also be extracted from natural sources. Plants are the major sources of these compounds. Ayurveda is based on compounds, derived from natural sources only[1,2]. It can be leaves, roots, flowers, stem, peels, or fruits of the plants. Our country is very rich in natural resources like fruits, vegetables, leaves, etc. of various herbs. From the past research, we have found that there are so many possibilities in the extraction of bioactive components derived from leaves of Indian medicinal plants and their animal testing [3, 4].

Mulberry is a highly useful plant of moraceae family. The size of this deciduous tree is about 6 to 9 m in height and widely cultivated for leaves, which are used for feeding the larva of a silkworm [5,6]. Quercetin, isoquercetin, gallic acid, clorogenic acid, tannins, choline, adenine, and trigonelline are also present in leaves [7,8].

The mulberry tree is propagated by cuttings, seeds, grafts, or budding and planted in the rainy season. Sandy and loamy soils are preferred for cultivation. The size of the seeds is extremely small. Seeds are dispersed by birds, jackals, and human beings [9,10,]. Generally, herbal medicines are very effective and standardization of these medicines are also important to determine its safety, purity, quality, and biological properties [34,35, 8,15].

The macroscopic and, microscopic characters, physico-chemical parameters, and fluorescence study were carried out under the standardization of the herbal drugs[11,12,39]. The sensory characters like color, taste, odor, consistency, shape, size, texture, and appearance of the surface are means to determine the quality, purity, and identity of herbal drugs[29,30,36,37]. Section cutting and microscopy of powder show the of calcium oxalate crystals, stomata, fibers, starch granules, trichomes, vessels, parenchyma, sclerenchyma, collenchymas, palisade cells, and stone cells helps in the identification of drugs[13,14,31]. Various parameters have been determined in the form of quantitative microscopy i.e. vein-islet number, stomatal number, stomatal index, vein-islet termination number, and palisade ratio helps in the identification of the herbal drug [32,33,41,38].

MATERIAL AND METHODS

Authentication of Plant:

Morus nigra plant leaves were collected from IET campus Lucknow.. The sample was authentified by NBRI Lucknow (U.P) as *Morus nigra* (accession No. is LWG - 100983).

Organoleptic Characters:

For the study of organoleptic characters the leaf is subjected to visual inspection to investigate the color, odor, taste and texture. A randomly selected leaf is used to determine the average length and width. The surface of the lamina is observed to recorded shape, size, venation, margin, and apex. The presence or absence of the petiole, base, and type of leaf has also been recorded[16,17].

Macroscopic Characters:

Morus nigra is a medium-sized tree, having a diameter of 0.5-1.0 m and 6 to 9 m in height. The mature trees trunk bark have greyish brown or reddish-brown and in form of irregular stripes. The bark has been separated by very shallow furrows[18].

Microscopical Characterization:
The microscopical characters have been observed with the help of transverse section of morus leaves. Safranin is used for staining the sections. After this, a drop of glycerin and thin section of the leaf has been placed in the center of the clean glass slide. A cover slip placed on the slide in absence of air bubble ensnared inside[16,19].

Powder Microscopy:
M. nigra leaves have been crushed into fine powder and clear with KOH diluted solution. A drop of glycerin has been placed in the center of the clean glass slide. Minute quantities of crude powdered drug put on the center of slide and place a cover slip properly to observed different features[16,40].

Fluorescence Investigation:
The herbal medicinal molecules absorb visible and UV light at specific wavelengths of 366 nm and 254 nm. Some selective chemical reagents used for getting fluorescence detection for the determination of crude drugs[16].

Physicochemical Parameters:
Various parameters are used for identification of sample such as moisture content, ash value, extractive value and foaming index [16,20].

Ash value determination:
The purity, as well as quality of a crude drug, may be analyzed with the help of a calculated amount of ash. The amount of silicates, carbonates, phosphates and oxalates have been estimated by this parameter. In this process, organic component is converted in carbon dioxide (CO_2). [16].

***Estimation of total ash*:**
As per IP 1996 guidelines, silicates, phosphates, carbonates and silica are the main components of the total ash. Two-gram of powdered leaves of *M. nigra* was taken and crude drug was incinerated at 170^0 C temperatures. The total ash has been estimated with the help of air-dried crude drugs[21].

***Estimation of acid insoluble ash*:**
The acid-insoluble ash has been obtained as residue on boiling total ash with hydrochloric acid for 5 minutes. This material was filtered, washed, incinerated and cooled. The quantity of ash (acid-insoluble) has been estimated with the help of dried crude drugs[20].

***Calculation of water-soluble ash*:**
It is obtained by total ash boiling with 25ml of distilled water only for 5 minutes. This resulting material was filtered, washed, incinerated followed by cooling in desiccators. The quantity of ash (water-insoluble) has been estimated with the help of dried crude drugs[21].

Calculation of extractive value:
Numerous organic solvents have been used to determine the presence of phytochemical compounds in our crude drug[20].

Estimation of alcohol soluble extractive value:
5 gm of the air-dried powdered drug has been macerated with 95% ethanol in an airtight conical flask for 24 hours. The sample has been shaken during the first 6 hours and then stand for 18 hours. Approximately 25 ml of the filtrate has been evaporated to dryness at 105^0C and weighed. The alcoholic extractive value (%) has been determined with the help of crude drug[16,21].

***Estimation of water-soluble extractive value*:**
5 gm powdered sample have been macerated with 100ml water in a conical flask and placed it for 24 hours. The sample has been shaken frequently during the first 6 hours and then stand for 24 hours. After filtration, the filtrate allows to dryness at 105^0C and weighed. The water-soluble extractive value (%) has been calculated by using the reference to the air-dried drug [16,21].

Determination of foreign organic matter:
For the calculation of foreign organic matter the drug sample, the presence of dust, molds, stones, animal excreta, soil, insects, should be negligible. Some amount of powered crude drug has been spread on glass sheet in the form of uniform thin layer. It can be detected either by magnifying glass or by necked eye. Undesired organic material has been separated through a sieve and the percentage of foreign organic material has been estimated[20,21].

Calculation of moisture content:
Some amount of crude drug taken into a porcelain dish and dried at 100-105 ^{0}C temperature for 5 hours and again weighing it. The process of dryness has been continued until to its constant weight. The loss in weight is usually recorded as moister content[16,21].

Analysis of foaming index:
1 g of the plant materials has been accurately weighed and mixed with 100 ml of boiling water in 500 ml conical flask and maintained the temperature at 80- 90^0C for 30 minutes. Then cool down sample and filtrate it into a volumetric flask (100 ml capacity). For dilution, a sufficient amount of water has been added to dilute. Now ten test tubes are used for decoction and marked from one to ten numbers. If the height of the foam in test tube is more than 1cm, the foaming index is more than one thousand[16].

Leaf Constants Estimation:
Surfaces of leaf has been investigated by removing of upper and lower surfaces (epidermal) then washed with potassium hydroxide solution and observed under a microscope for its stomatal number, stomatal index, vein termination number, vein islet number and palisade ratio. These measurements of leaves used to differentiate closely related species[22].

Stomatal number:
The potassium hydroxide solution has been used to clear the middle part of the leaf. By using forceps, the upper and lower epidermises have been peeled and placed on a glass slide. Defined area (1 mm^2) has been investigated with the help of camera lucida to count the number of stomata. Recorded the results in triplicate and estimated the mean value of stomatal count in every mm^2 area[22,23].

Stomatal index:
Upper and lower epidermis separated by forceps and placed on a slide. The micrometer and camera lucida has been used to drawn and counted in 1 mm^2 of stomata and epidermal cells in area 1.0 mm^2, respectively. Triplicate set of data have been recorded and mean value was calculated[22,23].

Palisade ratio:
The chloral hydrate solution has been used to clear the mid portion of the leaf (2 mm^2) and placed the sample on a slide and mounted it in glycerin water. To tracing the epidermal cells and the palisade cells, a camera lucida has been used. For the estimation of mean value, 25 groups of samples (leaves) were taken [22,23].

Vein-islet number:
Potassium hydroxide solution has been used to wash a section of *Morus* leaf by steaming for thirty minutes. The camera lucida has been set up and 1 mm^2 area of paper has been splitted with the help of stage micrometer. During the investigation of 2 mm^2 area of cleared leaf, veins have been found in 4 consecutive squares. Mean value have been calculated in 2mm^2 area[22,23].

Veinlet termination number:
Veinlet termination has also been estimated similarly as vein-islet number. During the investigation of 1mm^2 area, veins-islets have been found in 4 adjoining squares. Mean values and range have been calculated in 2.0 mm^2 area[22,23].

Pharmacological study:

Preparation of hydro-alcoholic extract
Morus leaves were powdered and packed in soxhlet apparatus. The extraction process has been done by using solvents such as ethanol (40%) and distilled water (60%) at 40^0C temperature[29,31]. The process of extraction was continued up to 22 cycles (approx 48 hours) when the soxhlet (siphon tube) become colorless. Drying process was done with the help of rotary evaporator after filtration and preserved in suitable size vials in the cold place[24].

***Animal selection*:**
Male SD rats (7-8 weeks old, 160±20 gm) have been taken for hypolipidemic activity. The rats were housed in cages (polypropylene) containing 23±2^0C temperature, humidity 50–60% and light intensity of 300 lux. Drinking water and food diet was provided to test animals (SD rats) to ad libitum[25].

***Hypolipidemic activity*:**
When the period of fasting of test animals (SD rats) become complete (one night), they were injected with single dose of triton-x-100 to make them hyperlipidemic (100 mg/kg in normal solution, saline in nature) intra peritoneally. The rats were divided into four groups with six animals in every group[26]. 300 mg/kg body weight of the freshly prepared leaves extract and standard drug Atorvastatin (10 mg/kg) were dissolve in normal saline solution have been given orally to SD rats.

Group 1- Normal control (normal saline solution)
Group 2- Freshly prepared solution of Triton-X-100 (100 mg/kg in normal saline solution) by intraperitoneal injection.
Group 3- Standard drug Atorvastatin(10 mg/kg) by oral administration.
Group 4- Treated orally with dose of 300 mg/kg hydroalcoholic extact of *M. nigra.*

***Collection of blood sample*:**
A small amount (2-4 ml) of blood was withdrawn at the end of one week from fasted (overnight) every SD rat. Ether was used as an anesthesia and blood was collected from retro-orbital venous plexus via capillary tube (glass) of every group[19]. Blood clotting was allowed by keeping it uninterrupted at normal atmospheric temperature, after that collected blood sample was centrifuged (10 min, 2.5x10^3 rpm) for separation the serum. Different parameters like triglycerides (TG), total cholesterol (TC), low-density lipoprotein (LDL) and (HDL) high-density lipoprotein were estimated by the analysis of this separated serum through standard methods[27,28].

***Statistical analysis*:**
The triplicate sets of data have been collected and one way analysis of variance (ANOVA) has been performed to analyze the data for getting results. The outcome has been analyzed in the form of mean ± SD.

RESULT AND DISCUSSION

Organoleptic Characters:
The leaf is subjected to visual inspection to investigate the color, odor, taste & texture.

Macroscopic characters:
The leaves of *Morus nigra Linn* are varied with their shape and size approximately 7 to 12 cm in length.
The leaves are simple, serrate shaped, sometimes divided into 1-2 lobes, thick, 3-nerved (Fig. 1(a)).
Its flowers are greenish-yellow monoecious with brown stigma branches.
Mulberry fruit has been found in oblong or an oval shape. The color of fruit become black after ripening, 1-2 centimeters long, it is opulently flavored (Fig. 1(b)).

Fig.1: Macroscopic characters of *Morus nigra* Linn (black mulberry) **(a)** Appearance of leaves **(b)** Appearance of fruits.

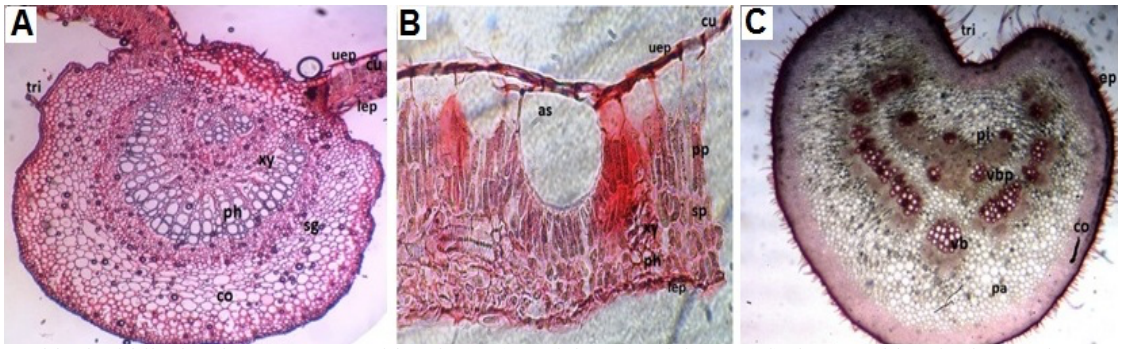

Fig.2: T.S of leaf midrib showing **(a)** upper epidermis (uep), cuticle (cu), trichome (tri), lower epidermis (lep), xylem (xy), starch grains (sg), phloem (ph), collenchyma (co). **(b)** T.S of lamina showing air space (as), cuticle(cu), upper epidermis (uep), spongy parenchyma (sp), palisade parenchyma (pp), phloem (ph), xylem(xy), lower epidermis (lep). **(c)** T.S of the petiole showing epidermis (ep), trichomes (tri), pith (pi), collenchyma (co), vascular bundles of pith (vbp), parenchyma (pa), vascular bundles (vb).

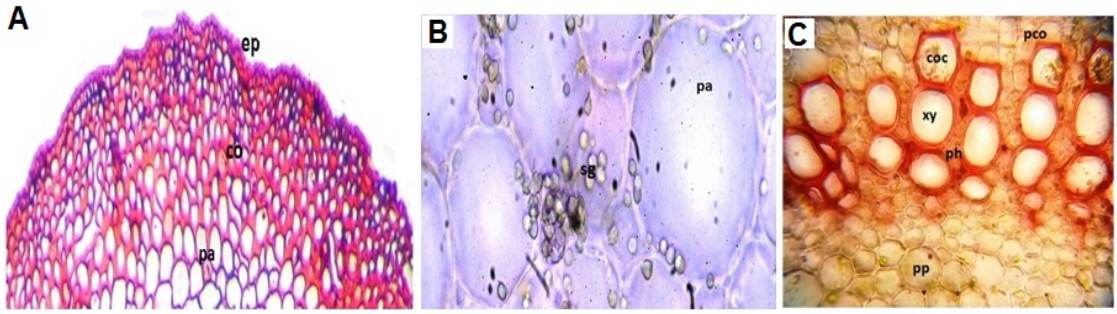

Fig.3: Cortex **(a)** epidermis (ep), collenchymas (co), parenchyma (pa). **(b)** Parenchymatous cells (pa) with starch granules (sg). **(c)** Vascular bundles in the pith zone showing calcium oxalate crystals (coc), pith collenchyma (pco), xylem (xy), pith parenchyma (pp), phloem (ph).

Microscopic characters:

Transverse Section via midrib of the mulberry leaf has epidermis (single-layered) along with some covering trichomes (Fig. 2(a)). Cortex has a thin region of collenchymas and narrow-walled parenchymatous cells. Crescent-shaped vascular bundles are found in the central region with phloem covering the xylem on the lower surface.

Upper and lower epidermis layers were observed in transverse section of lamina. Lower and upper epidermis layers are consisting of rectangular cell and arranged in tangential direction (in a single layer) with a thick cuticle. Below the upper epidermis layer, air space (large) has been found. Lamina has spongy parenchyma (5-10 layers) and palisade cells (2 layers). Loosely arranged parenchyma cells were found in spongy parenchyma (Fig. 2(b)).

T.S of petiole was oval in shape and a small groove appears on upper side. Single layered epidermis is found externally covered with a wide cuticle. Various thick-walled and long unicellular trichomes along with some glandular trichomes have been found on the exterior surface. Arrangement of various vascular bundles has been found in a ring shape (Fig. 2(c)). Xylem composed of tracheids, vessels, fibers and parenchyma. The phloem consists of sieve tubes, companion cells and phloem parenchyma. All cells are lignified and thick-walled.

The cortex contains a wide region of oval to circular lean wall of cells (parenchyma), on the other hand 6-7 thin layers of collenchyma cells (Fig. 3(a)). Parenchyma cells in the cortex region, consist of circular shaped starch grains (small in size) (Fig. 3(b)). Parenchyma cells (thin walled) and collenchyma cells are found in pith region while calcium oxalates crystals (rosettes) are found in large quantity (Fig. 3(c)).

Powder microscopy:

Some glandular trichomes and various uni-seriate covering trichomes are found in the mulberry leaf (Fig. 4(a-b)). Scalaryform xylem vessels are also observed in the powder microscopy of leaf (Fig. 4(c)). Stomata are paracytic type, upper epidermis shows less stomata in comparison to the lower epidermis (Fig. 4(d)).

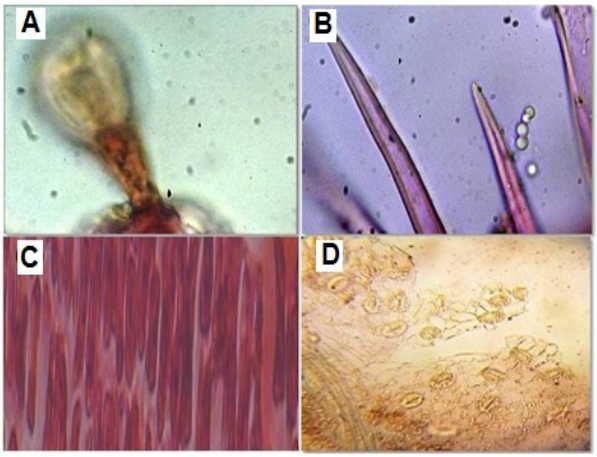

Fig.4: Powder microscopy of leaf **(a)** Glandular trichome **(b)** Covering trichomes **(c)** Scalaryform xylem vessel **(d)** Paracytic type Stomata.

Table 1: Fluorescence study of the sample

Visible light	Powder and used Chemicals	UV (254nm) Lamp	UV(366nm) Lamp
Green (light)	Dried powder	Green (dark)	Greenish brown
Green (yellowish)	NaoH (5%) + Powder	Brown	Green (dark)
Green (dark)	KOH (5%)+Power	Green (brownish)	Brown
Brown (blackish)	H_2SO_4 + Powder	Brown (greenish)	Dark brown
Red (light)	Conc. HCl+ Powder	Brown (reddish)	Brown
(Green) blackish	Conc.HNO_3+ Powder	Green (yellowish)	Greenish brown
Green (light)	Iodine solution +Powder	Green	Brownish-yellow
Brown (dark)	dil. Ammonia +Powder	Brown (greenish)	Green (dark)

Table 2: Parameters and leaf constants of the samples

Physico-chemical Parameters (%w/w)		Determination of Leaf constants	
Total ash	9.0	Stomatal number	21
Water-soluble ash	6.85	Stomatal index	20.34
Acid insoluble ash	3.52	Palisade ratio	22.45
Alcohol soluble extractive value	10.56	Vein-islet number	3
Water-soluble extractive value	3.82	Vein termination number	4
Foreign organic matter	0.48	-	-
Moisture content	10.12	-	-
Foaming index	100	-	-

Table 3: Effect of hydroalcoholic *Morus nigra* leaf extract on serum lipid level in SD rats

Groups	Normal Control	Diseased Control	Standard Control	*Morus nigra*
TC (mg/dl)	109.85±2.25	219.27±6.93	122.7±15.33	102.87±2.58
TG (mg/dl)	104.65±4.63	161.32±7.52	108.05±7.38	119.97±6.32
HDL (mg/dl)	29.32±4.49	19.37±3.33	27.27±4.90	25.12±1.43
LDL (mg/dl)	79.62±3.15	103.25±3.88	87.95±8.34	92.2±10.26

Fluorescence study:

This study has been done by using chemical reagents with samples in presence of visible light and UV light (366 nm and 254 nm). The fluorescence study of the samples has been shown in Table 1.

Physico-chemical parametric study of the samples and leaf constants:

The output values of all physico-chemical parameters of the samples such as total ash (9.0 %w/w), water-soluble ash (6.85 %w/w), acid insoluble ash (3.52 %w/w), Alcohol soluble extractive value (10.56 %w/w), water-soluble extractive value (3.82 %w/w), foreign organic matter (0.48 %w/w), moisture content (10.12 %w/w) and foaming index (100%w/w) are summarized in Table 2. The leaf constant values such as stomatal number (21), stomatal index (20.34), palisade ratio (22.45), Vein-islet number (3), Vein termination number (4) are also summarized. The results are summarized in Table 2.

Hypolipidemic activity:

The result shows that serum cholesterol level was increased in Triton X-100 (100 mg/kg) treated control group but high-density lipoprotein was decreased. Atorvastatin standard drug (10mg/kg) showed highly reduction in serum cholesterol level. Hydroalcoholic plant extracts (300 mg/kg) showed significant ($P < 0.05$) reduction in serum cholesterol level. Low-density lipoprotein (LDL), Triglycerides (TG) and total cholesterol (TC) were significantly reduced while high-density lipoprotein (HDL) was significantly increased (Table 3).

CONCLUSIONS:

In this article, we have discussed the hypolipidemic activity as well as pharmacognostical parameters like macroscopic characters, microscopic characters, and physicochemical parameters of leaves of *Morus nigra*. Evaluation of physicochemical parameters and leaf constants like extractive value, ash value, moisture content, foreign organic matter, foaming index, stomatal number, stomatal index, vein islet number, vein termination number and palisade ratio have been analyzed. These investigations help in standardization, quality control and authentification along with biological studied on medicinal plant *Morus nigra*.

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Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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