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Investigation of *in-vitro* antioxidant activity and heavy metals present in *Ficus mollis* leaves

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Abstract

The folklore medicinal plant leaves of Ficus mollis were selected for the study. We were performed for elemental analysis, i.e., macronutrients and toxic and heavy metal contents. All the values are within the safe limits. The plant specimens were successively extracted with solvents such as petroleum ether, Chloroform, ethyl acetate, and Ethanol. The preliminary phytochemical studies of FML confirmed the presence of flavonoids, tannins, steroids, glycosides, alkaloids, and triterpenoids. The present study also investigated FML antioxidant activity using in vitro methods. The EEFML showed significant antioxidant activity in all models studied compared with ascorbic acid. It possesses good activity in scavenging DPPH radical, Hydroxyl radical, Nitric oxide radical, and scavenging H2O2.Further investigation of individual Phyto compounds, their isolation, identification, and their role as free radical scavengers is needed.

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INTRODUCTION

Disease and death are problems that have engaged the attention of man from very early times. The primitive men did not know about medicine. They used those things they could procure most efficiently as therapeutic agents and remedial measures. But the Rig Veda, the oldest book in the man's library, supplies curious information. From Veda, it is learned that the Indo-Aryans used the 'Soma,' which is a plant product used as a medical agent, and it has a marvelous stimulating effect.

Also, from ancient civilizations, various parts of plants and animals were used to eliminate pain, control suffering, and counteract disease. By trial and error, primitive men must have acquired biological knowledge that helped determine which plants and animals possessed food value and which were to be avoided because they were unpalatable, poisonous, or dangerous. The healing

powers of certain herbs, roots, and juices were undoubtedly discovered in ancient times [1].

The use of herbal drugs in the traditional systems of medicine does not require marked modifications; either extract essence or powder may be required. But, for modern medical science, herbal drugs are subjected to several processes such as identification, isolation, purification, characterization, structural elucidation, and therapeutical evaluation. In every continent of the world, all the above works are successfully carried out, except in a few places where the versatility is deficient [2].

MATERIALS AND METHODS

List of chemicals used

In the current work, each solvent used is of analytical quality. Qualigen's Chemicals Pvt. Ltd., Rankem Chemicals Pvt. Ltd., S.D. Fine Chemicals Pvt. Ltd. Merck Chemicals Pvt. Ltd.

Other suppliers provided the necessary chemicals.Formalin, Glacial Acetic acid, Ethyl alcohol, Tert.butylalcohol, Paraffin wax, Toluidine blue, Saffranin, Iodine, Potassium iodide, Sodium hydroxide, Glycerin, Silica gel-G, Toluene, Ethyl acetate, Diethylamine, Formic acid, Chloroform, Acetone, n- butanol, Dragendorff's reagent, Vanillin, Conc. Sulphuric acid, Ammonia, Petroleum ether, Ethyl acetate, Ethanol.

Collection of plant material

Collection of *Ficus mollis Vahl* The plant specimens for the proposed Ficus mollis vahl study were collected from Tirumala hills, Andra Pradesh [3].

Elemental Analysis

An inductively coupled plasma optical emission spectrometer (ICP-OES) was used to analyze elements such as lead, palladium, mercury, potassium, magnesium, calcium, arsenic, and sodium [4].

Digestion and preparation of sample

After precisely measuring and adding 3 grams of the dry powdered material to the crucible, the temperature was steadily raised to 500–6000 degrees Celsius until colorless ash was produced. Ten milliliters of HNO3 were added to one gram of the ash mentioned above, which was then burned at 1500 degrees Celsius until the nitric acid content was cut in half. Five milliliters of nitric acid were left behind after adding 10 milliliters of the solution above once more and heating it to 2500 degrees Celsius. After the solution reached room temperature, it was heated to produce a dense fume, treated with three milliliters of perchloric acid. Then, it was run through an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) [5].

Using an inductively coupled plasma optical emission spectrometer (ICP-OES-BERTY Series II VARIAN) for elemental analysis [6]

ICP-OES determined the elemental analysis of digested samples. Analyses have been done on mercury, lead, palladium, arsenic, potassium, magnesium, and calcium. This technique added a homogenous liquid sample to a plasma where unbound atoms might release wavelength-specific emissions.

The unbound atoms reach the initial excited state. This spontaneous emission's intensity is quantified. The following formula can be used to determine the intensity of the radiated emission.

$$1cm = J/s/mx2 = W/mx2)$$

Macronutrients

Following standard protocol, sodium, potassium, and magnesium were quantified using an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES).

Toxic and heavy metal content

The toxic elements like arsenic, lead, palladium, and mercury were estimated using an inductively coupled plasma optical emission spectrometer (ICP-OES). All the observations confirm the safety of the preclinical and clinical studies [7].

Preparation of Extracts

The freshly collected leaves of Ficus mollis were cut into small pieces. Shade was dried and coarsely powdered, and 500 gm of plant material was packed in the Soxhlet apparatus and successfully extracted with petroleum ether and Water. Every time, the powdered components were dried in a hot air oven at a temperature below 50 °C before being extracted using the subsequent solvent. Ultimately, the extracts were concentrated using a Buchi R-114 rota evaporator and dried under a vacuum desiccator [8].

Solvents

Analytical grade reagents and solvents were utilized throughout the entire research.

The general approach for extraction of different extracts from coarse powder can be represented schematically.

Preliminary Phytochemical Screening

Test for Carbohydrates

Molisch's Test

Add a few drops of α-naphthol solution in alcohol to 2ml of the extract. Conc.H2SO4 was added after the test tube was shaken. Two liquids are mixed to form a violet ring. Confirm that carbohydrates are present.

Test for Reducing Sugars

Fehling's Test

Blend 1 milliliter of Fehling's A and B solutions with the extract and bring it to a boil for five to ten minutes. The precipitate turned brick crimson. Confirm that reducing sugar is present.

Benedict's Test

Add Benedict's reagent to a few milliliters of the extract and let it sit in a water bath for five minutes. The presence of reducing sugars is indicated by the emergence of brick-red, yellow, and green colors [9].

Test for Proteins and Amino Acids

Biuret Test

A few drops of 1% CuSO4 and 4% NaOH were added to 3ml of the extract. Proteins can be detected by their violet or pink color.

Millon Test

A few milliliters of Millon's reagent were added to three milliliters of the extract; the white precipitate shows the presence of proteins.

Test of Ninhydrin

Three drops of 5% Ninhydrin's reagent were added to 3 milliliters of the extract, and the mixture was cooked in a boiling water bath. The presence of amino acids is indicated by the purple or blueish color [10].

Test for Oils and Fats

Spot Examine

The stain was seen after applying a drop of the extract to the filter paper. The stain is still present, proving that fixed oils are present.

Test for Saponification

A small amount of different extracts and a drop of phenolphthalein were added separately, along with a few drops of 0.5N alcoholic KOH. The mixture was then cooked in a water bath for one to two hours. The development of soap or partial alkali neutralisation indicates the presence of fixed oils and fats.

Test for Steroids

Liebermann – Burchard Reaction

Add three milliliters of glacial acetic acid and acetic anhydride to each, and the extract is diluted in a few drops of Chloroform. Warm, then chilled, under the tap. Conc. H2SO4 was applied in drops along the test tube's sidewalls. An appearance of a reddish-green color indicates the presence of steroids.

Test for Glycosides

The extract was combined with a small amount of anthrone on a watch glass. After adding one drop of concentrated sulfuric acid, a paste was formed and slowly heated over a water bath. The dark green colour indicates glycosides

A. Warm 200 mg of the drug in a water bath while extracting it with 5 ml of diluted H2SO4. A 5% NaOH solution was used to neutralize the acid extract by the filter. After adding 0.1 ml of Fehling's Solutions A and B to make it alkaline, heat it for two minutes in a water bath. Take note of the red residue produced about Test B.

B. Warm the drug 200 mg in a water bath and extract it using 5 ml of H2O rather than H2SO4. To confirm that NaOH is present in the test mentioned above, add an equivalent volume of Water after boiling. Put Fehling's Solutions A and B in 0.1 ml increments until alkaline. It gets heated for two minutes in a water bath. The amount of red precipitate that formed should be noted. Make a comparison between Test A and Test B's precipitate formation amounts. The presence of glycosides is shown by the more significant amount of silt in Test A compared to Test B [11].

Test for Anthraquinones

Bontrager Test

After adding the extract and boiling and filtering, the diluted H_2SO_4, ether, filtered aqueous ammonia, or caustic soda were added. After shaking, anthraquinones are indicated by a pink, red, or violet color in the aqueous layer. If glycoside is detected, the test needs to be adjusted by first hydrolyzing it using hydrochloric acid.

Test for Coumarin Glycosides

A small specimen should be placed in a test tube, which should then be covered with filter paper soaked with diluted NaOH. Gently remove the paper, let it sit in a water bath for a few minutes, and then expose it to U.V. light. Green fluorescence shows the presence of coumarin glycosides.

Test for Saponins

Foam Test

The extract was mixed with a few milliliters of Water. Saponins are present when persistent foam is present.

Hemolytic Test

One drop of the extract and one drop of blood were added on a glass slide. The hemolytic zone shows saponins.

Test for Flavonoids

Shinoda's Test

A few minutes were spent boiling the extract after adding 5ml of 95% alcohol, 0.5g of magnesium turnings, and conc. HCl. A pink or red color indicates flavonoids.

Ammonia or a 10% NaOH solution was added to the extract along with alcohol. The presence of flavonoids is indicated by a dark yellow colour [12].

Zinc Hydrochloric Acid Test

This test solution is mixed with strong HCl and zinc dust. After a few minutes, the appearance of red shows the existence of flavonoids.

Test for Alkaloids

Dragendorff's Test

It was thoroughly shaken after adding a few drops of acetic acid and Dragendorff's reagent to the extract. An orange-red precipitate indicates the presence of alkaloids.

Mayer's Test

The extract was mixed with Mayer's reagent and a few drops of hydrochloric acid. A white precipitate indicates the presence of alkaloids.

Test for Tannins and Phenolic Compounds

A few milliliters of extract were mixed with a simple lead acetate solution. The presence of white precipitate indicates tannins.
A few drops of alcohol and ferric chloride were added to a few milliliters of the extract. A bluish-green or red color indicates the presence of phenolic compounds.

Test for Phytosterols and Triterpenoids

Liebermann–Burchard's test

After dissolving the extract in acetic anhydride, heating the mixture to boiling, let it cool, and then add 1 milliliter of pure sulfuric acid down the test tube's side. The presence of steroids, triterpenoids, and their glycosides is indicated by the red, pink, or violet color at the liquid-liquid junction.

Salkowski test

The chloroform extract is mixed with a few drops of sulfuric solid acid and agitated; the presence of triterpenoids is indicated by a golden yellow color, whereas the presence of steroids is shown by red in the lower layer.

Noller's Test

Tin and thionyl chloride were used to warm the extract. When terpenoids are present, the color turns pink [13].

Test for Gums and Mucilage

A test tube filled with alcohol was gradually filled with the extract while being continuously stirred. A precipitate's development suggests the existence of mucilages and gums.

Test for Resins

5–10 milliliters of acetic anhydride were added to the extract. Cooled and heated gently. 0.5 cc of H_2SO_4 was added to this. Violet quickly replaced the bright, reddish-red hue, signifying the presence of resins.

Antioxidant activity

Numerous antioxidants have been found in herbal plants. It has been proposed that a wide range of chemicals function as antioxidants. Numerous phenolic antioxidants, including tannins, coumarins, xanthones, flavonoids, and more recently, procyanidins, have been shown to scavenge radicals in a dose-dependent manner; as a result, they are thought to be prospective therapeutic agents for diseases caused by free radicals. The human body constantly produces reactive oxygen species (ROS) and free radicals, such as superoxide anion (O2-), hydrogen peroxide (H2O2), and hydroxyl radical (O.H.-), as a result of regular metabolic activity. These substances have been linked to the etiology of several diseases, including atherosclerosis, cancer, aging, and diabetes. The following in-vitro antioxidant investigations were conducted using extracts from ethanolic Ficus mollis leaves (EEFML) [14].

DPPH Radical Scavenging Activity

Using the 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) assay, the In-Vitro technique evaluated the antioxidant activity of ethanolic extracts of *Ficus mollis* leaves [15]. Three milliliters of DPPH in methanol were placed in a test tube with 20, 40, 80, 160, and 320 µg/ml of each milliliter of extract or standard. DPPH control absorbance value was found to be 0.875. The scavenging inhibition values were calculated using the formula mentioned below.

Scavenged (%) = [Abs (control) – Abs (standard) /Abs (control)] × 100

The Activity of Hydroxyl Radical Scavenging

Using Halliwell's modified approach, the hydroxyl radical scavenging capability was determined. Distilled deionized Water was used to express stock solutions of EDTA (1 mM), FeCl3 (10 mM), ascorbic acid (1 mM), H2O2 (10 mM), and deoxyribose (10 mM). The absorbance value of the control was found to be 0.954. The concentration of the sample needed to scavenge 50% of the sample's free radical is known as the IC50 value. IC50 values were calculated using the graphical presentation and compared with ascorbic acid, which was used as standard [16].

Scavenged (%) = [Abs (control) – Abs (standard) / Abs (control)] × 100

The Activity of Nitric Oxide Radical Scavenging

In an aqueous solution, sodium nitroprusside produces nitric oxide on its own. When dissolved oxygen and Water come into contact with the nitric oxide produced in this way, it transforms into nitric acid and nitrous acids. The absorbance value of the control was found to be 1.214. The scavenging inhibition of different concentrations of test and standard was calculated using the formula below. The concentration of the sample needed to scavenge 50% of the sample's free radical is known as the IC50 value. IC50 values were calculated in the graphical presentation and compared with those of ascorbic acid [17],

Scavenged (%) = [Abs (control) – Abs (standard) / Abs (control)] × 100

Hydrogen Peroxide Scavenging Activity

Hydrogen peroxide is the most minor reactive molecule among reactive oxygen species and is stable under physiological pH and temperature without metal ions. The absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The absorbance value of the control was found to be 0.986. The IC50 value is the sample concentration required to scavenge 50% free radical of the sample. IC50 values were calculated using the graphical presentation and compared with ascorbic acid, used as a standard.

% Scavenging [H2O2] is equal to [Abs (standard) - Abs (control) / Abs (control)]. × 100

Chromatographic Studies

Chromatography is essential for separating and estimating different components of crude plant drugs.

Thin layer chromatography

TLC was identified because of its selectivity, ease, and flexibility in handling small amounts of material. When a mixture of components is spotted on a TLC plate, the compounds that are readily soluble but not strongly absorbed rise with the solvent, while those that are not so soluble but more strongly absorbed rise lower, resulting in compound separation. The discovery of active

principles in medicinal plants is critical in the phytochemical investigation of crude plant extracts, especially regarding their potential pharmacological effects. This can be achieved using thin-layer chromatography [18].

RESULTS AND DISCUSSION

Elemental Analysis

The harmful effects of heavy metals are becoming increasingly important in modern times. Ingestion of these may be helpful or hazardous, depending on the type of ingredient present. The dangerous components may acquire cumulative or hereditary toxicity, as well as be carcinogenic.

Macronutrients

Macronutrients such as sodium, potassium, magnesium, and calcium were quantified using an Inductively Coupled Plasma Optical Emission Spectrometer (ICP- OES) using a standard protocol [**Table 1**].

Table 1 Macronutrient content

Metals	FML	
	Crude Drug	Ethanolic Extract
Magnesium	2.6542	1.4794
Potassium	11.2914	8.2056
Sodium	38.0224	13.5713
Calcium	43.3214	12.983

Each value is represented in ppm.

Toxic and heavy metal content:

Toxic elements such as arsenic, lead, palladium, mercury, and cadmium were measured using an Inductively Coupled Plasma Optical Emission Spectrometer [**Table 2**].

Table 2 Toxic and Heavy Metals Content

Metals	FML	
	Crude Drug	Methanolic extract
Arsenic	0.0124	0.0045
Lead	0.1452	0.0436
Palladium	0.0231	0.0274
Mercury	0.1325	0.0198
Cadmium	0.0159	0.0021

Each value is represented in ppm.

All the observations confirm the safety of the preclinical and clinical studies.

Extractions

The percentage yields of the extractives of stem bark and leaves of *Ficus mollis Vahl*., as well as roots of *Datura fastuosa Linn*., were observed [**Table 3**].

Table 3 Extractive values in Different solvents

Extractives	% yield of FML
Petroleum ether	3.8
Chloroform	3.2
Ethyl acetate	4.8
Ethanol	13.6

Preliminary phytochemical studies

The Preliminary phytochemical studies of extractives of stem bark and leaves of Ficus mollis Vahl. The roots of *Datura fastuosa* Linn. **Table 4**.

Antioxidant activity

The following Tables 5 to 9 document the results of the investigation into the antioxidant activity of the ethanolic extract of Ficus mollis leaves:

DISCUSSION

The target-rich and lead scenario raises questions about ethnopharmacology and drug discovery using natural materials. Ethnopharmacology is where a lot of contemporary medications originate. Research and practice in traditional and integrative health sciences are favorably trending worldwide. Standard methods for finding new drugs include combinatorial chemistry, chemical synthesis, genomics, chemical biology, and uncertainty. Nevertheless, the novel techniques include ethnopharmacology, reverse pharmacology, holistic, system biology, and personalized medicine. Substantial patterns indicate that the predominant direction in pharmaceutical research is shifting from single molecules or single targets to combinations and many targets. A proper reverse pharmacology approach to drug research is made possible by ethnopharmacology's experimental foundation and expertise, which extends from clinics to labs. The effectiveness of this method is determined via validation. An inventive discovery engine for better, safer, cheaper, and more successful therapies will be formed by combining a golden triangle of traditional wisdom, modern medicine, and modern science with a systems orientation.

Table 4 Initial phytochemical screening of FML extracts

Phytochemical Constituents	Petroleum ether Extract FML	Chloroform Extract FML	Ethyl acetate Extract FML	Ethanolic Extract FML
Alkaloids	-	-	++	+
Carbohydrates	-	-	-	-
Flavonoids	-	-	++	++
Glycosides	+	+	-	+
Tannins/phenols	+	+	+	++
Protein	-	-	-	-
Steroids	-	-	+	-
Saponins	-	-	+	-
Triterpenoids	+	+	++	+
Fats and Oils	-	-	_	-
Anthraquinones	_	_	_	-
Gums and mucilage	_	_	_	-
Resins	-	-	_	-

+: Indicate the presence of Phytochemical Constituents;
-: Indicate absence of Phytochemical Constituents

Table 5 DPPH Assay Scavenging Profile

Concentration (µg)	Absorbance		% scavenging inhibit	
	EEFML	Ascorbic Acid (std)	EEFML	Ascorbic Acid (std)
20	0.724	0.724	17.24±2.12	17.15± 3.12
40	0.635	0.627	27.34±2.32	27.34± 2.32
80	0.503	0.486	42.51± 0.78	44.43± 1.56
160	0.390	0.383	55.34± 1.57	56.27± 2.51
320	0.205	0.192	76.52 ±4.31	78.02± 0.86

Table 6 : Hydroxyl Radical Assay Scavenging Profile

Concentration (µg)	Absorbance		% scavenging inhibit	
	EEFML	Ascorbic Acid (std)	EEFML	Ascorbic Acid (std)
20	0.779	0.772	18.25±0.76	19.02 ±2.52
40	0.684	0.673	28.26 ±1.76	29.45± 4.31
80	0.534	0.512	44.01± 1.78	46.3± 2.36
160	0.436	0.400	54.27± 2.45	58.01± 2.11
320	0.206	0.194	78.32± 3.01	79.61 ±1.76

The proportion of macronutrients within the prescribed ranges, including Mg, K, Na, and Ca. Arsenic, lead, palladium, mercury, and cadmium are examples of heavy and dangerous metals that are present in moderation and guarantee the safety of plants as drugs. Ficus mollis leaves were extracted into petroleum ether, Chloroform, Ethanol, and ethyl acetate in that order. Flavonoids, tannins, steroids, triterpenoids, and phenolic substances were verified to be present in Ficus mollis vahl. Leaves based on preliminary phytochemical analyses. The ethanol extract of Ficus mollis leaves at different concentrations (20–320µg/ml) was evaluated for their ability to scavenge free radicals. Free radical scavenging was found to occur in a dose-dependent manner.

One of the free radicals that is frequently used to assess a compound's or plant extract's initial capacity to scavenge radicals is DPPH. A stable free radical in an aqueous or methanolic solution is the DPPH radical. It becomes stable as a diamagnetic molecule by accepting an electron or a hydrogen radical. By visible spectroscopy, DPPH has a significant absorption maximum at 517 nm due to its odd electron. In the presence of extract, the antioxidant activity as determined by the radical's odd electron capacity pairs off (hydrogen

Table 7 Nitric oxide assay Scavenging Profile

S.No	Method of Assay	EEFML (µg/ml)	Ascorbic acid (µg/ml)
1	DPPH Scavenging Assay	123.35	105.21
2	Hydroxyl Radical Assay	121.50	102.45
3	Nitric oxide assay	112.30	90.25
4	Hydrogen peroxide Assay	118.82	97.63

Table 8 Hydrogen Peroxide Assay Scavenging Profile

Concentration (µg)	Absorbance		% scavenging inhibit	
	EEFML	Ascorbic Acid (std)	EEFML	Ascorbic Acid (std)
20	0.993	0.979	18.14 ±0.12	19.31± 0.89
40	0.884	0.856	27.12± 1.5	29.41± 3.56
80	0.678	0.629	44.13± 1.56	48.13± 2.06
160	0.519	0.516	57.32± 1.75	57.42± 1.43
320	0.265	0.241	78.13 ±4.24	80.13± 1.54

Table 9 Antioxidant IC50 values in different Invitro models

S. No	Concentration (µg)	Absorbance		% scavenging inhibit	
		EEFML	Ascorbic Acid (std)	EEFML	Ascorbic Acid (std)
1	20	0.806	0.796	18.16± 3.25	19.24± 2.51
2	40	0.687	0.688	30.25± 1.41	30.13± 3.02
3	80	0.540	0.517	45.23 ±5.14	47.52± 1.32
4	160	0.412	0.400	58.21± 4.31	59.42± 2.05
5	320	0.183	0.176	81.41 ±3.51	82.13± 1.75

donor). The absorption strength decreases when it pairs off, and the ensuing decolorization is stoichiometric about the quantity of electrons absorbed. The ability to carry out this reaction makes certain substances antioxidants, making them radical scavengers. **Table 5** displays the findings of the DPPH Scavenging. These data were additionally displayed graphically. A highly reactive species that arises in biological systems, hydroxyl radicals have been linked to free radical pathology and are capable of causing damage to nearly every molecule present in live cells. This species also can quickly initiate the process of lipid peroxidation, which abstracts hydrogen atoms from unsaturated fatty acids. It will further injure cells by upsetting the biomembrane. Scavenging hydroxyl radicals is critical. **Table 6** displayed the extracts of leaves and stem bark's hydroxyl radical scavenging values. The basis for the nitric oxide radical scavenging assay method is the following: at physiological pH, sodium nitroprusside in aqueous solution spontaneously produces nitric oxide, which reacts with oxygen to form nitrite ions, which may be measured with the Griess reagent. Reduced generation of nitrite ions results from scavengers of nitric oxide competing with oxygen. **Table 7** displays the results of the assay for nitric oxide radical scavenging. **Table 8** also displayed the values for hydrogen peroxide radical scavenging. **Table 9** displays the values obtained from the IC50 calculations for all antioxidant activity techniques.

CONCLUSION

This research study revealed that folk medicinal plants have profound antibacterial and antioxidant activity, which was experimentally proved, and that they are safe to use as folk medicine. These plant specimens have a lot of scope for further studies to establish more pharmacological activities. The FML may have good anti-fungal activity and wound healing due to the presence of triterpenoids. Antidiabetic and hepato-protective activity is also possible because of the presence of activities in other closely resembling species of Ficus.

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