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HEPATOPROTECTIVE EFFICACY OF METHANOLIC EXTRACT OF LEAVES OF *CISSAMPELOS PAREIRA* IN ACETAMINOPHEN INDUCED TOXICITY ON HEP G2 CELLS

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ABSRACT

Objectives: To investigate the hepatoprotective efficacy of most active extract of leaves of *Cissampelos pareira* in acetaminophen induced toxicity on Hep G2 cell lines. **Materials and Methods**: Phytochemical studies were carried out using three different solvents like hexane, ethyl acetate and methanol. The antioxidant activity of different extracts was studied by in vitro assay using DPPH and IC50 value was determined. HPTLC analysis was also carried out. Hepatoprotective efficacy of methanolic extract of *C. pareira* in acetaminophen induced Hep G2cell lines were analysed using MTT assay. **Results**: Preliminary phytochemical analysis revealed the presence of phenol, flavonoid, alkaloid, glycosides, saponins, steroids, carbohydrates, proteins and aminoacids. From the different extracts methanolic extract showed high antioxidant activity. HPTLC analysis of methanolic extract showed the presence of flavonoid. The cytotoxic studies revealed that methanolic extract of leaves of *Cissampelos pareira* at a concentration of 12.5µg/ml showed maximum hepatoprotective action in acetaminophen induced Hep G2 cells. **Conclusion:** The results suggest a hepatoprotective action for methanolic extract of leaves of *Cissampelos pareira* in acetaminophen induced Hep G2 cells due to its antioxidant properties.

KEYWORDS: Hepatoprotection, Cytotoxicity, Acetaminophen, Hep G2 cells, *Cissampelos pareira.*

1. INTRODUCTION

Medicinal herbs and plants play an important role in world health. In all over the world the use of medicinal plants has attained a major role in health system. Medicinal plants are not only used for treatment of diseases but also for maintaining good health and conditions. The reason for depending herbal medicine for primary health care is because of their better cultural acceptability, better compatibility and adaptability with the human body and pose lesser side effects. Medicinal plants have been playing an essential role in the development of human culture. Many of the modern medicines are produced from medicinal plants.[1]

Man has been using herbal and plant products for combating diseases, times immemorial. The Indian subcontinent is enriched by a variety of flora, both aromatic and medicinal plants. Numerous types of herbs have been well recognized and catalogued by botanists. This extensive flora has been greatly utilized as a source of many drugs in the Indian traditional system of medicine. All plants produce chemical compounds as part of their normal metabolic activity. These include primary metabolites, such as sugars and fats, found in all plants and secondary metabolites found in smaller range of plants, some useful ones found only in a particular genus or species. Plant secondary metabolites have been of interest to man for along time due to their pharmacological relevance.[2]

The liver performs the normal metabolic homeostasis of the body as well as biotransformation, detoxification and excretion of many endogenous and exogenous compounds, including pharmaceutical and environmental chemicals.Drug-induced hepatotoxicity is a major cause of iatrogenic diseases, accounting for one in 600 to one in 3500 of all hospital admissions.[3] There is an acute necessity of reliable hepatoprotective drugs in modern medical practice.Plants and natural products have been used traditionally worldwide for the prevention and treatment of liver disease. Scientific research has supported the claims of the medicinal efficacy of several of these herbal compounds, as evidenced from the voluminous work on their hepatoprotective potentials.[4]

Hepatic disease is a united term for an entire group of trouble that afflict the tissues, structures and cells of the human liver. Large number of important functions is performed by liver, so there are lots of opening for somewhat to go incorrect. One of the most common causes of liver disease is inflammation, which often results from abuse of alcohol, poor diet or even malnutrition.[5] Drug induced liver damage or liver dysfunction is the most important

health crisis that challenges not only medical personnel but also the pharmaceutical field and drug control board.

According to the United States acute liver failure study group, drug induced liver injury accounts for more than 50% of acute liver failure, including hepatotoxicity caused by over dose of acetaminophen (39%) and idiosyncratic liver injury triggered by other drug.[6] Hepatic-cell injury caused by various toxic chemicals (certain antibiotic, chemotherapeutic agents, carbon tetrachloride (CCl_4), thioacetamide (TAA) etc.), excessive alcohol consumption and microbes.[7]

1.1: HEPATOTOXICITY

Hepatotoxin is a toxic chemical substance which damages the liver. Toxic liver injury produced by drugs and chemicals may virtually mimic any form of naturally occurring liver disease. The use of drugs had generated some noxious effects on patients. Among the various inorganic compounds producing hepatotoxicity are arsenic, phosphorus, copper and iron. The organic agents include certain naturally occurring plant toxins such as pyrrolizidine alkaloids, myotoxins and bacterial toxins. Liver injury caused by hepatotoxins, such as carbon tetra chloride (CCl4), ethanol and acetaminophen, is characterized by varying degrees of hepatocyte degeneration and cell death via either apoptosis or necrosis.

In addition to specific treatment for a given hepatotoxin, the general strategy for prevention and treatment of the damage includes reducing the production of reactive metabolites of the hepatotoxins, using anti-oxidative agents and selectively targeting therapeutics to Kupffer cells or hepatocytes for ongoing processes, which play a role in mediating a second phase of the injury.[8]

1.2: ACETAMINOPHEN

Paracetamol, also known as acetaminophen or APAP, is a medication used to treat pain and fever. It is typically used for mild to moderate pain. In combination with opioid pain medication, paracetamol is also used for more severe pain such as cancer pain and after surgery. Acetaminophen, taken in overdose can cause severe hepatotoxicity and nephrotoxicity.[9] The mechanism of action of paracetamol is not completely understood. Drug induced liver damage

(hepatotoxicity) results not from paracetamol itself, but from one of its metabolites, *N*-acetyl-*p*-benzoquinoneimine (NAPQI). NAPQI decreases the liver's natural antioxidant glutathione and directly damages cells in the liver, leading to liver failure.

1.3: LIVER DISORDERS

Liver diseases are among the most serious ailment. They may be classified as acute or chronic hepatitis (inflammatory liver diseases), hepatosis (non inflammatory diseases) and cirrhosis (degenerative disorder resulting in fibrosis of the liver). Liver diseases are mainly caused by toxic chemicals (certain antibiotics, chemotherapeutics, peroxidised oil, aflatoxin, carbon-tetrachloride, chlorinated hydrocarbons etc.), excess consumption of alcohol, infections and autoimmune/disorder.[10]

1.4: Hep G2 CELL LINE

Hep G2 is a perpetual cell line consisting of human liver carcinoma cells, derived from the liver tissue of a 15 year old Caucasian male who had a well differentiated hepatocellular carcinoma. The morphology of Hep G2 cells is epithelial and they have 55 chromosome pairs. Hep G2 are adherent, epithelial-like cells growing as monolayers and in small aggregate. Because of their high degree of morphological and functional differentiation *in vitro*, Hep G2 cells are a suitable model to study the intracellular trafficking and dynamics of bile canalicular and sinusoidal membrane proteins and lipids in human hepatocytes *in vitro*. This can be important for the study of human liver diseases that are caused by an incorrect subcellular distribution of cell surface proteins. Hep G2 cells and their derivatives are also used as a model system for studies of liver metabolism and toxicity.

1.5: PLANT PROFILE

Cissampelos pareira

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Order	:	Ranunculales
Family	:	Menispermaceae
Genus	:	*Cissampelos L.*
Species	:	*C. pareira*

Cissampelos pareira (Family Menispermaceae), is a sub-erect or climbing herb, known as ambastha or malathangi in Indian traditional medicine.[11] There are 37 plant species summarized under this botanical name. All these species are found in all over the world but only one species occur in India. The plant is common in orchards, hedges, parks and gardens on moist soils distributed throughout tropical and subtropical India, ascending up to an altitude of 2000 m, either creeping or twining around other plants. It is also common on the hilly tracts along watercourses.

1.6: PLANT DESCRIPTION

The plant is a climbing shrub, 2 - 5m high with a thickened root. Leaves are green, have an orbicular shape 7-14 cm in diameter. Some leaves are round, ovate or oblong, and some may even be linear. Stem length ranges from one to six feet (0.3-1.8 m), they are normally hairless but can be pubescent.[12] Flowers are green and unisexual; pedicel up to 2mm long. Roots, cylindrical, 1-1.5 cm in diameter, light brown to yellowish in colour.[13]

1.7: SECONDARY PLANT METABOLITES

Secondary plant metabolites are biosynthetically derived from the primary metabolites and their distribution in plant kingdom is restricted.[14] The secondary compounds include alkaloids, terpenoids, flavonoids, steroids and volatile oils. Attempts are being made on the researchof producing phytochemicals and also to discover new compounds from plants for using as pharmaceuticals. The alkaloids, tannins, flavonoids and phenol compounds play a major role in preventing a number of chronic diseases by a definite physiological action on the human body like anti-inflammatory, anti-thrombotic, anti-oxidant, hepatoprotective and anticarcinogenic activities.[15]

1.8: ANTIOXIDANTS

Free radicals are unstable chemical species naturally occur in the body as a result of exogenous chemical and endogenous metabolic process in the human body. Reactive oxygen species (ROS) including superoxide radicals, hydroxyl radicals, singlet oxygen and hydrogen peroxide are often generated as byproducts of biological reaction or from exogenous factors.[16] ROS produced by ultraviolet light, ionizing radiation, chemical reactions and metabolic processes have a wide variety of pathological effects such as DNA damage, carcinogenesis and various degenerative disorders such as cardiovascular diseases, ageing and neuro- degenerative diseases.[17],[18],[19]

Recent reports indicated that there is an inverse relationship between dietary intake of antioxidant rich foods and the incidence of human diseases.[20] Therefore the commercial production development and utilization of plants as sources of antioxidants to enhance health and food preservation is of current interest. Antioxidant based drugs and formulations for the prevention and treatment of complex diseases like Alzheimer's disease, liver diseases and cancer have appeared during last three decades.[21] Recent studies shown that a number of plant products including polyphenols, terpenes and various plant extracts exerted an antioxidant action. [22], [23],[24]

1.9: STUDIES ON *CISSAMPELOS PAREIRA*

1.9.1: Phytochemical studies

Phytochemical studies have shown that the major chemical components of this herb are alkaloids, flavonoids, tannins, volatile oils and glycosides. Pelosine, deyamittin, cissamine, cycleanine and l-curine have been reported from the roots. Root bark was reported to contain menismine, pareirine and hayatinine.The rhizomes contain hayatine, hayatidine, d-4''o-methylbebeerine, L-bebeerine, isochondrodendrine, dicentrine, dehydrodicentrine, insularine. The rhizomes and leaves contain cycloanine.[25]The leaves contain nuciferine, bulbo-carpine, corytuberine, laudanosine and magniflorine from the leaves and stems. The leaves also contain 70- 75% galacturonicacid , little amount of neutral sugars a flavonoid quercetin and a saturated fatty acid, eicosanoic acid.[26]

1.9.2: Medicinal uses

The leaves of the velvet leaf has been highly valued for many years in traditional Chinese and Indian ayurvedic medicine for its medical qualities. A number of pharmacological and biological activities including anti- diarrhoeal, anti-protozoal, antileukemic, curariform, anti-inflammatory, anti-fertility, anthelmintic, antinociceptive, antiarthritic, antiulcer, antioxidant, anti-hemorrhagic, hepatoprotective, memory enhancing, antihyperglycemic, cardioprotective, immunomodulatory, antidengue, diuretic, antiplasmodial, anti-tumour, evaluation of toxicity.

1.9.3: Therapeutic uses

Velvet leaf is used in the treatment of chronic non-healing ulcers and sinuses. It is also used in the treatment of chronic skin diseases and in the treatment of poisonous bites. Its anti-inflammatory activity has been investigated with encouraging results. infusion of the bitter rhizome, and sometimes of leaves and stems, to cure gastro-intestinal complaints such as diarrhoea, dysentery, ulcers, colic, intestinal worms and digestive complaints, and also

urogenital problems such as menstrual problems, venereal diseases, infertility, uterine bleeding and threatening miscarriage. Juice form macerated leaves and stem is mixed with a little water and used as anti-conjunctivitis or as a treatment for sore eyes. Juice from macerated leaves and stem is used an as an anti-inflammatory.Leaves and stem are macerated in water an used as an anti-infective agent.

1.9.4: Reported pharmacological studies

SI.NO	Activities	Extract	Part used
1	Antioxidant	Ethanolic	Root[27]
2	Antifertility	Hydro- alcoholic	Leaves[28]
3	Chemopreventive	Hydroalcoholic	Root[29]
4	Anti-haemorrhagic	Aqueous	Leaves[30]
5	Antinociceptive & Anti-arthritic	Ethanolic	Root[31]
6	Anti- inflammatory	Ethanolic	Aerial part[32]
7	Gastro protective	Ethanolic	Root[33]
8	Cardio protective	Ethanolic	Root[34]
9	Anti- diarrheal	Ethanolic	Root[35]
10	Hepatoprotective	Hydro-alcoholic	Root[36]
11	Memory enhancing	Hydro-alcoholic	Root[37]
12	Anti- hyperglycemic	Methanol	Root[38]

As there were no previous reports available for the hepatoprotective activity of the drug, the present study was undertaken to study the phytochemical analysis and hepatoprotective activity of methanolic extract of leaves of *Cissampelos pareira* in acetaminophen induced toxicity on Hep G2 cells with a view to provide a scientific evidence.

2. MATERIALS AND METHODS

2.1: PLANT PREPARATION

Samples used in the study were leaves of *Cissampelos pareira* collected during the months of January to March. Plant material was washed several times with water and allowed to shade dry at room temperature and authenticated.The voucher specimes (BC 09) were preserved in the Department.

2.2: CELL LINE

Hep G2 (Liver Hepatic cells) cell line was purchased from NCCS Pune were maintained in Dulbecco's modified eagles media (HIMEDIA) supplemented with 10% FBS (Invitrogen) and grown to confluency at 37ºC in a humidified 5% CO_2 in a humidified atmosphere in a CO_2 incubator (NBS Eppendorf, Germany).

2.3: CHEMICALS

All chemicals for the study were purchased from Merck, Mumbai, India, Himedia, India and Sisco Research Laboratories (SRL), India).

2.4: INSTRUMENTS

CO2 Incubator - (Eppendorf, Germany),
Phase Contrast Microscope - (Olympus, JAPAN with Optika Pro 5 Camera)
MTT - (Sigma Aldrich M5655)
ELISA Reader - (Erba, Germany)
Colorimeter - (Systronics, India)
Centrifuge - (Remi, India)

2.5: PREPARATION OF LEAF EXTRACTS BY DIFFERENT SOLVENTS

50g of dried powder of the *Cissampelos pareira* was subjected to successive cold extraction using three solvents based on their increasing polarity hexane, ethyl acetate, and methanol for 72 h at a temperature not exceeding the boiling point of solvent. 100ml of each solvent used for extraction. Vigorous shaking is required for proper extraction. Finally filter the extract using whatman no 1 filter paper. Note down the weight of each bottle prior to the drying of the extract and after drying too. Calculate the weight of the extract from the difference. The extracts were concentrated to dryness under reduced pressure and controlled temperature in a rotavapor. The yield of methanolic extract of *Cissampelos pareira* was noted.The dried extracts were stored in a clean sample bottles unti further use.

Percentage yield of extract = $\frac{(W1X100)}{W2}$

W1= weight of extract residue obtained after solvent extraction, W2= weight of powder taken

2.6: PRELIMINARY PHYTOCHEMICAL SCREENING

Preliminary phytochemical screening was carried out for hexane, ethyl acetate methanol and water, for the detection of phytoconstituents as per the standard conventional protocols.[39]

2.7: PHYTOCHEMICAL TESTS

Phytochemical examinations were carried out for all the extracts as per the standard methods.[40]

1. Test for alkaloids

a)Mayer's test: To 1 ml of extract added 2ml of Mayer's reagent (Potassium mercuric iodide). Formation of a cream colored precipitate indicated the presence of alkaloids.

b)Wagner's test: To 1 ml of extract added 2ml of Wagner's reagent (Iodine in potassium iodide). Formation of brown/ reddish precipitate indicated the presence of alkaloids.

c)Dragendroff's test: To 1 ml of extract 1ml of Dragendroff's reagent (solution of potassium bismuth iodide) was added. Formation of orange – red precipitate indicated the presence of alkaloids.

2. Test for Flavanoids

a) Lead acetate test: Extract was treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless3 on addition of dilute acid, indicated the presence of flavanoids.

b)Alkaline reagent test: Extract was treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicated the presence of flavanoids.

c)The extract was treated with sodium hydroxide; formation of yellow colour indicated the presence of flavones.

3.Test for phenolic compounds and tannins

a)1ml of the test solution was mixed with basic lead acetate solution and the formation of white precipitate indicated the presence of tannins and phenolic compounds.

b)To 1ml of the extract, ferric chloride solution was added. Formation of a dark blue or greenish black colour product confirmed the presence of phenolic compounds and tannins.

c)Strong potassium dichromate solution was added to the test extract, yellow colour precipitate confirmed the presence of tannin and phenolic compounds.

4. Test for glycosides

a) Legal's test: 1 ml of the extract was treated with sodium nitroprusside in pyridine and sodium hydroxide. Formation of pink to blood red colour indicated the presence of cardiac glycosides.

b) 5 ml of aqueous extract was mixed with 2ml of glacial acetic acid contains 1 drop of ferric chloride solution carefully added to1ml of concentrated sulphuric acid. (The concentrated sulphuric acid is seen underneath the mixture). Formation of brown ring indicated the presence of cardiac glycosides.

5. Test for saponins

a)Froth test: 1 ml of the extract was diluted to 20 ml with water and was shaken in a graduated cylinder for 15 min. Formation of 1 cm layer of foam indicated the presence of saponins.

b)Foam test: 0.5g of extract was shaken with 2ml of water. If foam produced persists for ten minutes it indicated the presence of saponins.

6. Test for phytosterols

a)Salkowski's tests: 1 ml of the extract was treated with few drops of concentrated sulphuric acid, shaken and allowed to stand. A ppearance of golden yellow colour indicated the presence of triterpenes.

b)Libermann Buchard's test: 1 ml of the extractwas treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled. Concentrated Sulphuric acid was added, a violet ring at the zone of contact with the supernatant. Green or violet colour indicated the presence of sterols and triterpenes.

7. Test for carbohydrates

Extracts are dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

a)Molisch's test : 1 ml of the filtrate was treated with 2 drops of alcoholic α- naphthol solution in a test tube. Formation of a violet ring at the junction indicated the presence of carbohydrates.

b)Benedict's test: 1 ml of the filtrate was treated with 5 ml of Benedict's reagent and heated gently. Orange red precipitate indicated the presence of reducing sugars.

c)Fehling's test: 1ml of the filtrate was hydrolyzed with dilute hydrochloric acid, neutralized with alkali and heated with Fehling's A and B solutions. Formation of red precipitate indicated the presence of reducing sugars.

8. Test for fixed oils and fats

a)Spot test: Pressed a small quantity of extract between the filter paper. Oil stain on paper indicated the presence of fixed oils.

b)Saponification test: Few drops of 0.5 N alcoholic potassium hydroxide were added to a small quantity of extract along with a drop of phenolphthalein. The mixture was heated on a water bath for 1-2 h. Formation of soap or partial neutralization of alkali indicated the presence of fixed oils and fats.

9. Detection of proteins and aminoacids

a)Xanthoproteic test: The extracts were treated with few drops of concentrated nitric acid. Formation of yellow colour indicated the presence of proteins.

b)Ninhydrin test: To the extract, 0.25% w/v Ninhydrin reagent was added and boiled for a few minutes. Formation of blue colour indicated the presence of amino acod.

c)Biuret test: To 2ml of the extract added 2 ml of 10% sodium hydroxide solution followed by 2 drops of 0.1% copper sulphate solution. Formation of purple violet colour indicated the presence of proteins.

2.7: *IN VITRO* ANTIOXIDANT STUDIES

The plant extract were subjected to free radical scavenging activities such as DPPH and nitric oxide radical scavenging assays. The plant extracts were subjected to quantification of phytoconstituents such as flavanoids, total phenolic content and total antioxidant capacity.

2.7.1: DPPH radical scavenging activity[41]

The determination of the free radical scavenging activity of each of the crude extract was carried using the DPPH (1-diphenyl-2-picrylhydrazil) assay with a slight modification. Various concentrations of 50, 100, 200 and 400 µg/ml of sample extracts in methanol were prepared. 1.0 ml of a 0.1 Mm DPPH in methanol was added to 2.5 ml solution of the extract or standard, and allowed to stand at room temperature in a dark chamber for 30 min. The change in colour from deep violet to yellow was then measured at 518 nm on a spectrophotometer. The percentage of inhibition was calculated.

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

The total phenolic content in the methanol extract was determined by Folin-Ciocalteu method.[42] Total flavonoid content was measured with the aluminium chloride colorimetric assay.[43] The antioxidant activity of the extract was evaluated by the phosphomolybdenum method.[44]

2.8: HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC)

Sample applied on the HPTLC plate as a spot. Ethyl acetate: Methanol: Water at the ratio of 10:1.35:1 used as mobile phase. Mobile phase drawn through the stationary phase capillary action. Samples are separated in to their components which remain in their position on the layer after the mobile phase has been evaporated and visualized the bands.

Rf value=(Distance moved by solute front)/(Distance moved by solvent front)

2.9: *IN VITRO* HEPATOPROTECTIVE STUDIES

Cell viability was determined by use of the micro culture tetrazolium technique (MTT. It is a colourimetric assay that measures the reduction of yellow [3-(4,5- dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The viability of cells were evaluated by direct observation of cells by Inverted phase contrast icroscope and followed by MTT assay method.[45], [46]

2.9.1: Cytotoxicity Evaluation

After attaining sufficient growth Acetaminophen (20µM) was added to induce toxicity and incubated for one hour, prepared extracts in 5% DMEM were five times serially diluted by two fold dilution (100µg, 50µg, 25µg, 12.5µg, 6.25µg in 500µl of 5% DMEM) and each concentration of 100µl were added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO_2 incubator.

2.9.2: Cytotoxicity assay by direct microscopic observation

Entire plate was observed at an interval of each 24 hours; up to 72 hours in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observation were recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

2.9.3: Cytoxicity assay by MTT method

Fifteen mg of MTT was reconstituted in 3 ml PBS until completely dissolved and sterilized by filter sterilization. After 24 hours of incubation period, the sample content in wells were removed and 3 0µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37ºC in a humidified 5% CO_2 incubator for 4 hours. After the incubation period, the supernatant was removed and 100µl of MTT Solubilization Solution (DMSO was added and the wells were mixed gently by pipetting up and down in order to solubilize the formazan crystals). The absorbance values were measured by using microplate reader at a wavelength of 540 nm.

The percentage of growth inhibition was calculated using the formula.

$$\text{Percentage viability} = \frac{\text{mean OD samples}}{\text{mean OD of control group}} \times 100$$

2.10: STATISTICAL ANALYSIS

Results were expressed as mean ± S.E and all statistical comparisons were made by means of one way ANOVA test.

3. RESULTS

3.1: EXTRACTION IN DIFFERENT SOLVENTS

The result of extractive yield of powdered leaves of *Cissampelos pareira* in different solvents (hexane, ethyl acetate and methanol) obtained by successive extraction were shown in Table 1.

Table 1: Extractive yield of leaves of *Cissampelos pareira* in different solvents.

Sl. no.	Extract	Weight of powder(g)	Extractive value (g)	% yield (g)	Appearance
1	Hexane	15	0.25	1.67	Sticky, light green
2	Ethyl Acetate	15	0.81	5.4	Semisolid, green
3	Methanol	15	1.15	7.67	Semisolid, dark green

3.2: PHYTOCHEMICAL SCREENING

Preliminary phytochemical analysis of *Cissampelos pareira* revealed the presence of different types of secondary metabolites in hexane, ethyl acetate and methanol fractions as shown in Table 2. The phytochemical screening in the present study has revealed the presence of carbohydrates, glycosides, proteins, amino acids, steroids, saponins, phenols, flavonoids and alkaloids.

Table 2: Phytochemical screening of *Cissampelos pareira.*

Constituents	Hexane	Ethyl Acetate	Methanol
Alkaloids	+	+	+
Flavonoids	+	+	+
Phenolics	+	+	+
Tannins	-	-	-
Glycosides	-	+	+
Fats and oils	+	+	+
Steroids	+	-	-
Saponins	+	+	+
Carbohydrates	+	+	+
Proteins & amino acids	+	+	+

(+) sign indicates the presence of constituents; (-) sign indicates the absence of constituents

3.3: *IN VITRO* ANTIOXIDANT STUDIES

3.3.1: DPPH radical scavenging activity

The hexane, ethyl acetate and methanol extracts of *Cissampelos pareira* exhibited a significant dose dependent inhibition of DPPH activity. Methanol extract showed maximum scavenging activity. Ascorbic acid was used as standard. Percentage (%) scavenging activity was plotted against log concentration and from the graph IC_{50} value was calculated. Results given in Table 3.

Table 3: DPPH radical scavenging activity of different extracts of leaves of *C.pareira.*

Conc. (µg/ml)	% DPPH radical scavenging activity			
	Hexane	Ethyl Acetate	Methanol	Ascorbic acid
50	25.76±0.03	27.29±0.21	30.31±0.08	19.69±0.41
100	39.39±0.11	33.34±0.17	50.76±0.02	51.52±0.31
200	67.42±0.011	49.24±0.12	69.67±0.14	68.18±0.28
400	75.76±0.09	65.91±0.03	84.85±0.31	87.88±0.11
IC_{50}µg/ml	173	243	125	147

Values are mean ± SD (n=3)

3.3.2: Estimation of phytoconstituents

The methanolic extract of *Cissampelos pareira (*MECP) showed highest radical scavenging ability when compared to other extracts. Also the methanol extract of plant contained almost all phytochemicals. Hence MECP was used for further *in vitro* studies.

3.3.3: Determination of total phenolic, flavonoid and antioxidant activity of *MECP*

MECP showed a high total antioxidant activity, phenolic contents and total flavonoid contents. The results are given in Table 4.

Table 4: Total phenolic, flavonoids and antioxidant activity of Cissampelos pareira.

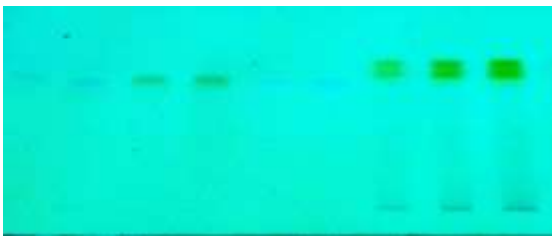
Extract	Total Phenolic content (mgGAE/g dry extract)	Flavonoids(mg QE/g dry extract)	Total antioxidant activity (mg ascorbic acid/g dry extract)
Methanol	20.57 ± 0.29	9.12 ±0.04	184 ± 3.72

Values are mean ± SD (n=3)

3.4: HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC)

Qualitative analysis of HPTLC of MECP showed presence of flavonoid. For HPTLC catechin, quercetin, reserpine were used as the standards. Ethyl acetate: Methanol: Water (10:1.35:1) was used as mobile phase. When MECP was analysed by HPTLC revealed the presence of flavonoids when compared with the standards catechin, quercetin and reserpine

"Fig.1."



UV radiation

Visible light

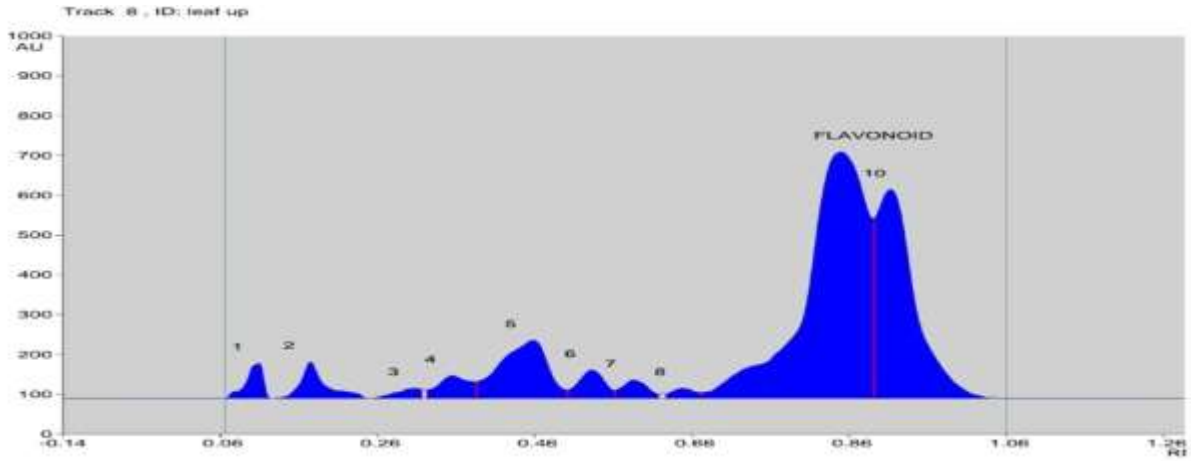


Fig. 1: Chromatogram of HPTLC

Peaks	Start position	Start height	Maximum position	End position	Area %	Assigned substance
1	0.07Rf	0.5AU	0.11Rf	0.12Rf	1.96%	Unknown*
2	0.13Rf	1.2AU	0.17Rf	0.24Rf	2.87%	Unknown*
3	0.25Rf	0.3AU	0.31Rf	0.31Rf	0.83%	Unknown*
4	0.32Rf	20.8AU	0.35Rf	0.38Rf	2.41%	Unknown*
5	0.38Rf	41.8AU	0.46Rf	0.50Rf	9.29%	Unknown*
6	0.50Rf	21.0AU	0.53Rf	0.56Rf	2.48%	Unknown*
7	0.56Rf	19.8AU	0.58Rf	0.62Rf	1.56%	Unknown*
8	0.63Rf	11.0AU	0.65Rf	0.67Rf	0.78%	Unknown*
9	0.67Rf	14.3AU	0.85Rf	0.89Rf	0.91%	Flavanoid
10	0.89Rf	52.3AU	0.91Rf	1.03Rf	6.92%	Unknown*

3.4*: IN VITRO* HEPATOPROTECTIVE STUDIES

MTT assay of MECP showed that the hepatoprotective activity was greater at concentration 12.5 µg/ml. Cells incubated in acetaminophen showed a decrease in cell viability. When Hep G2 cells incubated in acetaminophen (20mM) for 1 hr was treated with MECP (Various concentrations for 24 hrs) showed that an increase in cell viability upto a concentration of

12.5µg/ml. When concentration of sample increases it induced cell toxicity and showed a decrease in cell viability "Fig.2".

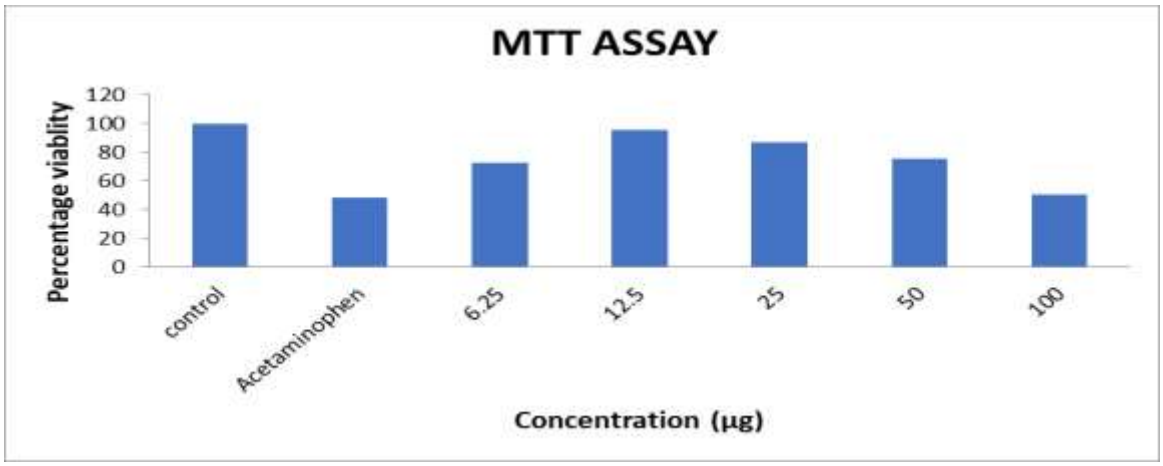
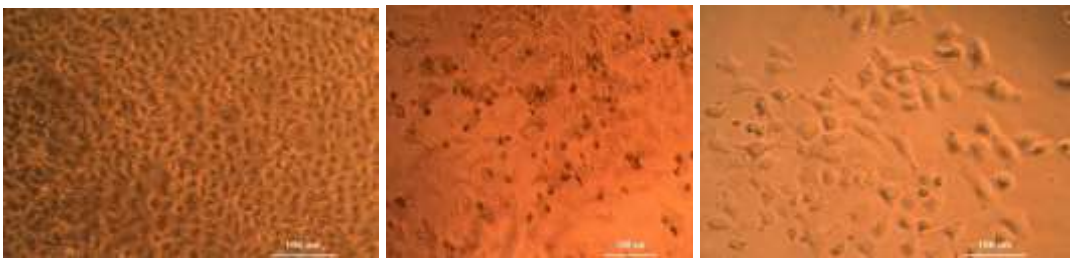
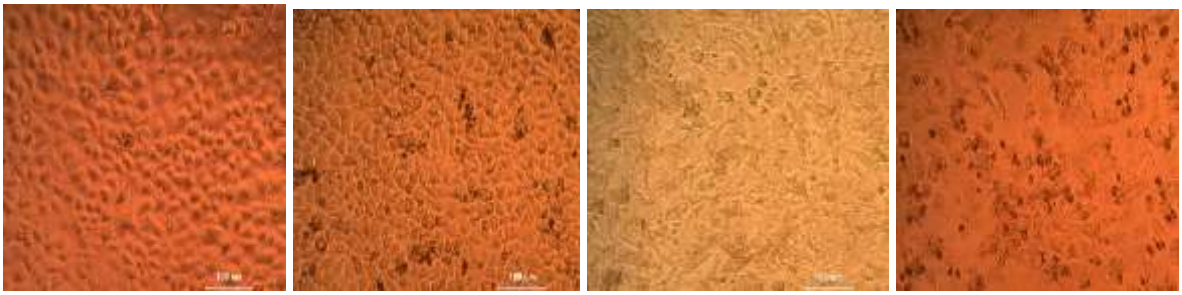


Fig.2: Hepatoprotective activity of MELCP in acetaminophen induced Hep G2 cell lines.

Methanol extract of Cissampelos pareira leaves showed increased viability at 12.5µg/ml concentration. As shown in figure 3 percentage of viable cells remained 95.27% at concentration of 12.5µg/ml even when cells treated with acetaminophen for 1hr. But when doses were increased, the percentage of viable cells was decreased. These result indicated that MECP had a hepatoprotective effect at concentration of 12.5µg/ml "Fig.3".



(A) **(B)** **(C)**



(D) **(E)** **(F)** **(G)**

Fig. 3: Photomicrograph of liver tissues.

(A) Control (B) Acetaminophen induced cells (C) Acetaminophen + MELCP (6.25µg/ml) (D) Acetaminophen+ MELCP (12.5µg/ml) (E) Acetaminophen + MELCP (25µg/ml) (F) Acetaminophen + MELCP (50µg/ml) (G) Acetaminophen + MELCP (100µg/ml)

4. DISCUSSION

The liver diseases are mainly caused by toxic chemicals (certain antibiotics, chemotherapeutics, peroxidised oil, aflatoxin, carbon tetrachloride, acetaminophen, chlorinated hydrocarbons, etc.) Paracetamol (PCM) also known as acetaminophen, taken in overdose can cause severe hepatotoxicity and nephrotoxicity.[47] There are four phases of acute paracetamol toxicity: Preclinical, hepatic injury, hepatic failure, and recovery.[48] Hep G2 cells and their derivatives are also used as a model system for studies of liver metabolism and toxicity.[49]

The phytochemical screening in the present study revealed the presence of carbohydrates, glycosides, proteins, amino acids, steroids, phenolics, tannins, flavonoids, saponins, terpenes and alkaloids. Most of the phytochemical constituents were present in the extracts of methanol, ethyl acetate, chloroform and hexane. Steroids were found only in the hexane extract. There were several works in connection with the antioxidant properties of phenolic compounds present in medicinal plants.[50]

Antioxidant that scavenges free radicals played an important role in various disorders. Studies showed that a number of plant products including polyphenols, terpenes and various plant extracts exerted an antioxidant action. The alkaloids, tannins, flavonoids and phenol compounds play a major role in preventing a number of chronic diseases by a definite physiological action on the human body like anti-inflammatory, anti-thrombotic, anti-oxidant, hepatoprotective and anticarcinogenic activities.[51]

In the present study MECP showed higher activity. The radical scavenging assays were decreased in the following order: methanol > ethyl acetate > hexane. The hexane, ethyl acetate and methanol extracts of *Cissampelos pareira* exhibited a significant dose dependent inhibition of DPPH activity. The MECP showed highest radical scavenging ability when compared to other extracts. MECP showed a high total antioxidant activity, phenolic contents and total flavonoid contents.

HPTLC method were used for the separation of phytoconstituents in MECP. Qualitative

analysis of HPTLC showed the presence of flavonoids in the sample when compared with the standards catechin, quercetin and reserpine. As antioxidants, flavonoids have been reported to be able to interfere with the activities of enzymes involved in reactive oxygen species generation, quenching free radicals, chelating transition metals and rendering them redox inactive in the Fenton reaction.[52] Due to the presence of this compound it showed a higher antioxidant activity.

Hepatoprotective activity was studied using MTT assay. Since the reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells.. MTT assay of MECP showed that the hepatoprotective activity is greater at concentration 12.5 µg/ml. Cells incubated in acetaminophen showed a decrease in cell viability. When concentration of sample increases it induced cell toxicity and showed a decrease in cell viability. Result indicated that MECP had a hepatoprotective effect at concentration of 12.5µg/ml.

5. CONCLUSION

Cissampelos pareira belonging to the family Menispermaceae is a perennial climbing herb commonly known as malathangi.Its leaves possesses diverse number of pharmacological activities and has long been used in traditional medicine in many countries. Phytochemical analysis of leaves of C. pareira revealed the presence of carbohydrates, glycosides, proteins, aminoacids, steroids, saponins, phenols, flavonoids and alkaloids. The present study also showed the antioxidant activity of leaves of *C. pareira* in various extracts like hexane, ethyl acetate and methanol. So methanolic extract is a richest source of natural antioxidants. Due to this reason it also possess greater pharmacological activity.

The antioxidant activity of various extracts of leaves of *C. pareira* analysed by compared with standard ascorbic acid. From the investigation it is understood that methanolic extract showed greatest free radical scavenging activity, so it is used for further studies.

HPTLC analyses of MECP were also analysed. HPTLC analysis revealed the presence of flavanoid. Catechin, reserpine and quercetin were used as standards for the analysis of the compounds.

Methanolic extract of *C. pareira* against acetaminophen induced toxicity in Hep G2 cell lines were also investigated by MTT assay. Over dosage of acetaminophen causes acute liver

damage. Acetaminophens (20mM) were treated with cell lines for 1hr, observed a decrease in cell viability. MECP samples were incubated for 24 hrs in acetaminophen treated cell lines showed a hepatoprotective action at a concentration of 12.5µg/ml. Beyond this concentration shows a sample toxicity to the cell lines and causes a decrease in cell viability.

Thus the present study on *Cissampelos pareira* concluded that the leaves of plant had antioxidant activity as well as a hepatoprotective efficacy against acetaminophen induced toxicity in Hep G2 cell lines. These properties of the extract is due to the combined effect of phytoconstituents like alkaloid, phenols and flavonoids. This study provides evidence for the plant's potential as a natural antioxidant and hepatoprotective agent, highlighting its potential for development as a hepatoprotective agent.

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