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ISOLATION AND CHARACTERIZATION OF FUNGAL ENDOPHYTIC FRACTIONS ASSOCIATED WITH LEAVES OF *MORINGA OLEIFERA* LAM. FOR *IN VIVO* HEPATOPROTECTIVE ACTIVITY IN CCl_4 INDUCED RATS

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ABSTRACT

The study of endophytes among the various microbial systems develops into a significant area of research for its significance in agriculture and medicine. Endophytes are tiny organisms that coexist symbiotically with robust plant tissues to live there. The goal of the study was to isolate, characterize, and screen the fungal extracts for *in vitro* antioxidant and *in vivo* hepatoprotective activity from the leaves of *Moringa oleifera*. One fungal endophyte, designated as MOL-1, was isolated and fermented to produce chloroform, ethyl acetate and n-butanol, (MOLC, MOLEA, MOLnB) extracts. *In vitro* free radical scavenging activity was carried by 2, 2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl radical and reducing power. MOLEA and MOLnB showed significant free radical scavenging activity. MOLEA and MOLnB (50 & 100 mg/kg) reversed the increased biochemical parameters such as serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), serum alkaline phosphate (SALP), total bilirubin, and direct bilirubin, as compared to CCl_4 induced group (***$p<0.0001$). MOLEA and MOLnB (100 mg/kg p.o) also restored the lipid peroxidation (LPO), superoxide (SOD) and catalase (CAT) levels. Endophytic extracts showed liver protection and can be

further utilized for pharmacological functions. MOL-1 was recognized as an isolate of *Rhizopus stolonifer* AR1 by PCR sequential analysis.

Keywords: ***Moringa oleifera*, *Rhizopus stolonifer AR1*, Endophytic fungi, Hepatoprotective, Antioxidant**

INTRODUCTION:

Different kinds of liver damage are thought to be caused by irresistible etiologically distinct triggers, such as severe hepatotoxic synthetic preparations and infections. In addition, the pathophysiology of free radicals and the oxidative damage they cause have been the subject of ongoing research in this extreme area of endophytic research. The discovery of the mechanism by which powerful liver toxins like CCl_4, paracetamol, and others reported works, also showed that free radical hepatotoxicity plays a pathophysiological role **[1]**. One of the few possible mechanisms, such as inflammatory cytokines, reactive aldehydes, covalent metabolite binding, and lipid peroxidation, has been blamed for the danger CCl_4 poses. As a result, each of the aforementioned systems contributes to the multifactorial process of CCl_4 toxicity **[2, 3]**. Regulating the roles of superoxide dismutase, catalase, and glutathione peroxidase enzymes can eliminate the generated free radical. One of the 14 species in the family Moringaceae known as Drumstick is *Moringa oleifera*. Because of its high nutritional value, *Moringa oleifera* is utilized in a variety of countries **[4]**. The whole plant is used to treat schizophrenia and eye conditions. It also has antipyretic properties, strengthens cells, and kills free radicals **[5]**. Antihepatotoxic properties of alcoholic concentrates derived from *Moringa oleifera* flowers and roots **[6]** were investigated.

Endophytes are microscopic organisms or actinomycetes that live in the productive tissues of plants and secrete a variety of bioactive compounds that are valuable to plants and useful to humans **[7]**. They become valuable compounds for the food industry, as anti-infectives, medicinal or drug substances, or both **[8, 9]**. One of the defining characteristics of microorganisms is the presence of secondary metabolites **[10]**. The isolated endophytic microbes from various medicinal plants showed various pharmacological activities, including antibacterial **[11]**, antimicrobial **[12]**, immunomodulatory and antioxidant effects **[13]**, antifungal activities **[14]**, anticancer **[15]**, hepatoprotective actions **[16]**. Endophytes also produce new phytochemical compounds with various biological activities, including taxol **[17]**, campothecin **[18]**, phyllanthin and hypophyllanthin **[19]**.

This study was carried out to isolate, characterize, and screen crude extracts for antioxidant effects and antihepatotoxic

activity in CCl_4 induced hepatotoxicity because there is no existing literature on this plant.

METHODS:

Plant authentication

Moringa oleifera Lam. leaves were collected near Dharwad in the Indian state of Karnataka. Dr. S. S. Hebbar, Botanist at Government Pre-University College in Dharwad, India, identified and authenticated the Plant. A specimen copy was kept in the Pharmacognosy department (SETCPD/Ph.cog/herb/15-6-2017).

Isolation of Endophytes

Moringa oleifera leaves were thoroughly washed and dried under running tap water to remove any foreign particles attached to them. They were then rinsed with running tap water for 10 minutes before sterilizing with 70% ethanol for 1 minute, 1% sodium hypochlorite (NaOCl) for 1-2 minutes, and 70% ethanol again. It is then cleaned by running it through two rows of sterile distilled water. Following surface sterilization, the leaves were cut into 1 cm pieces and placed on a plate containing potato dextrose agar (PDA) medium with 150 mg/L streptomycin to suppress fungal contamination. The plates were incubated for 7-14 days at 25°C-27°C until the fungal mycelium appeared. Fungal colonies were randomly selected from the plates, tested for purity, and grouped based on colony morphology. A pure endophytic fungus, MOL-1 (endophytic fungus of *Moringa oleifera* leaves), was chosen for the study **[20]**.

Fermentation and extraction

MOL-1 purified isolates were fermented separately in a 3000 mL Erlenmeyer flask containing 600 mL of potato dextrose broth. For 21 days, the flask was incubated at 25°C-27°C. Following the 21 days incubation period, 500 ml of chloroform was added to the flask and left overnight. It was then homogenized at 4000 rpm for 30 minutes to separate the mycelium from the broth before being vacuum filtered through Whatman filter paper. The resulting aqueous phase was partitioned three times with equal volumes of ethyl acetate. After ethyl acetate extraction, the aqueous phase was partitioned twice with equal volumes of n-butanol. The chloroform, ethyl acetate and n-butanol extracts of MOL-1 were dried using a vacuum rotary evaporator (Superfit Rotavap, PBU-6) and weighed **[21]**.

Preliminary Phytochemical Test

A preliminary phytochemical analysis of MOL-1 extracts; chloroform (MOLC), ethyl acetate (MOLEA), and n-butanol (MOLnB) was performed to identify the presence of primary and secondary metabolites **[22]**.

Phenotypic Identification

Colony Morphology; Lactophenol cotton blue staining was used to examine colonies grown on the slant. The properties of the

organisms were compared to those of known organisms from the literature **[23]**.

Molecular Characterization by PCR sequential analysis

Using a genomic DNA extraction kit, genomic DNA was extracted from the given organism (Bhat Biotech Ltd. Bangalore, India). Amplification of the 16s rRNA gene was carried out with the help of forward and reverse primers. For the amplification, a Master cycler thermal cycler (DNA-AMP Bhat Biotech) was used. BLAST N was used to compare the sequences to the non-redundant NCBI database. For the most similar sequences, the expected value and e values were recorded. CLUSTAL W2 was used to align ten similar neighbors. MEGA5 software was used to generate a phylogram from the resulting multiple alignment file **[24, 25]**.

***In vitro* free radical scavenging activity**

Reaction with DPPH radical

At various concentrations, the DPPH free radical scavenging activity of all MOLC, MOLEA, and MOLnB was tested **[26]**.

Reaction with hydroxyl radical

Hydroxyl radicals were generated using a F enton reaction, and the hydroxyl raical scavenging capacity was measured using the deoxyribose method **[27]**.

Determination of reducing power

The power of MOLC, MOLEA, and MOLn B was reduced using the method described by Oyaizu1986 **[28]**.

Hepatoprotective activity

MOLEA and MOLnB were chosen and tested for *in vivo* hepatoprotective activity in CCl_4 induced hepatotoxicity based on the results of free radical scavenging activity.

Animals

Albino wistar rats weighing 150-200 g were used in the study and were collected Aditya Biosys, Tumkuru, Karnataka, collected the inbred rats. For experimental purposes, they were housed in the animal house of SET's College of Pharmacy in Dharwad, under controlled conditions of temperature (23°C-25°C), humidity (50%), and 12 hour light dark cycles. Prior to the study, they were acclimated for 7 days. The animals were then divided into groups at random and housed individually in disinfected polypropylene cages with sterile rice husk as bedding. They had unlimited access to standard pellets as their staple diet and water.

Acute toxicity studies

The acute oral toxicity of MOLEA and MOLnB in Swiss albino mice was investigated. The animals were fasted for 12 hours prior to the experiment, given a single dose of extracts dissolved in 5% gum acacia, and mortality (short term toxicity) was monitored for up to 48 hours. The next

animal dose was determined using OECD Guideline 420 based on short-term toxicity.

Experimental design [29]

Animals were divided into following groups of six animals in each group (n=6).

Group I	Normal control, rats were administered p.o., a single daily dose of 0.5% Tween-80 (1 ml) on all 5 d and olive oil (1 ml/kg) s.c.
Group II	CCl_4 control, Rats treated with CCl_4 (2 ml/kg i.p. in olive oil)
Group III	Rats treated with Silymarin (100 mg/kg p.o) all 5 days and a single dose of CCl_4 (2 ml/kg) s.c., on day 2 and 3.
Group IV	Rats treated with MOLEA (50 mg/kg p.o), all 5 days and a single dose of CCl_4 (2 ml/kg) s.c., on day 2 and 3,
Group V	Rats treated with MOLEA (100 mg/kg p.o), all 5 days and a single dose of CCl_4 (2 ml/kg) s.c. on day 2 and 3.
Group VI	Rats treated with MOLnB (50 mg/kg p.o), all 5 days and a single dose of CCl_4 (2 ml/kg) s.c. on day 2 and 3.
Group VII	Rats treated with MOLnB (100 mg/kg p.o), all 5 days and a single dose of CCl_4 (2 ml/kg) s.c. on day 2 and 3.

During treatment, the rats were maintained on normal diet and water *ad libitum*. On 6th day, the animals were sacrificed, blood was collected by retro-orbital bleeding under mild ether anesthesia, centrifuged (3000 rpm for 15 min), and the serum was subjected to biochemical analysis. The liver was excised and placed in 10% formalin solution for histopathological examination. Liver homogenate was prepared to determine levels of endogenous enzymes.

Biochemical parameters

The separated serum was analyzed spectrophotometrically for AST, ALT, ALP, total and direct bilirubin, using diagnostic kits from Swemed Diagnostics, Bengaluru, India.

Measurement of enzymatic and non-enzymatic antioxidant parameters

Tissue preparation

The animals were sacrificed and transcardially perfused with ice cold saline. The entire liver was perfused *in situ* with ice cold saline before being excised, patted dry, and weighed immediately. Using a Teflon-glass homogenizer, a 10% liver homogenate was prepared separately with ice cold saline EDTA (Yamato LSG LH-21, Japan). The homogenate was used to calculate the amount of lipid peroxidation. The liver homogenate was centrifuged for 10 minutes at 10,000 rpm, and the pellet was discarded. The supernatant was centrifuged again for 1 hour at 4°C at 200 rpm. The obtained liver supernatants were used to calculate non-enzymatic antioxidants (lipid peroxidation) as well as enzymatic antioxidants (catalase and superoxide dismutase).

Lipid peroxidation

A standard procedure was used to estimate the concentration of thiobarbituric acid reactive substances (TBARS) in the liver homogenate. In brief, the homogenate was incubated at 95°C for 15 minutes with 15% TCA, 0.375% TBA, and 5N HCl before

being cooled, centrifuged, and the absorbance of the supernatant measured at 532 nm against an appropriate blank. Using the formula, the amount of lipid peroxidation was calculated and expressed as TBARS (moles) per gram of tissue **[30]**.

SOD assay

1 ml of 50 mM sodium carbonate, 0.4 ml of 24 m NBT, and 0.2 ml of 0.1 mM EDTA were added to 0.5 ml of liver homogenate. 0.4 ml of 1 mM hydroxylamine hydrochloride was added to start the reaction. The absorbance was measured at 560 nm at zero time, then again after 5 minutes at 25ºC. SOD activity was expressed as the amount of enzyme required to prevent NBT reduction by 50%. The specific activity was measured in units per mg of protein **[30]**.

Catalase

To 1.95 ml of 10 mM H_2O_2 in 60 mM phosphate buffer (pH=7.0), 0.05 ml of the liver homogenate was added and rate of degradation of H_2O_2 was followed at 240 nm/min. Catalase content in terms of U/mg of protein was estimated from the rate of decomposition of H_2O_2 using the formula

$$k=2.303/ \Delta t \times \log (A3/A4)\ s\text{-}1$$

A unit of catalase is defined as the quantity which decomposes 1.0 µmole of H_2O_2 per min at pH=7.0 at 25°C, while H_2O_2 concentration falls from 10.3 to 9.2 mM [30].

Histopathological studies

Individual animals' livers were excised, fixed in 10% buffered neutral formalin, and fixed in bovine broth. Standard micro technology was used to further process them for paraffin embedding. Photomicroscopic examination of liver sections stained with alum-hematoxylin and eosin revealed histopathological changes **[31]**.

Statistical evaluation

The data were expressed as Mean±S.E.M. Statistical comparisons were performed in one-way ANOVA followed by Tukey's t-test using Graph Pad Prism version 5, USA.

RESULTS:

Endophytic fungi MOL1 was isolated from *Moringa oleifera* leaves **(Figure 1)**. The percentage yield of MOLC, MOLEA and MOLnB was found to be 2.4%, 4.3% and 6.4% respectively.

Preliminary phytochemical screening

The qualitative chemical analysis of MOLC and MOLEA and MOLnB revealed the presence of flavonoids, glycosides, alkaloids, tannins, phytosterols as secondary metabolites.

DPPH assay

The IC50 values of MOLC, MOLEA and MOLnB were found to be 51.68 µg /ml, 41.74 µg /ml and 53.86 µg/ml respectively where as IC50 value for ascorbic acid was found to be 33.72 µg /ml. All the extracts showed significant scavenging activity **(Figure 2)**.

Hydroxyl radical Assay

In a Fe3+-EDTA-Mannitol and H_2O_2 reaction mixture, the extract and standard mannitol inhibited hydroxyl radical mediated deoxyribose degradation. MOLC, MOLEA, and MOLnB IC_{50} values were determined to be 347.69 µg/ml, 144.75 µg/ml, and 152.30 µg/ml, respectively. The IC_{50} value for standard mannitol was 165.2 µg/ml. As a result, when compared to MOLC, MOLEA and MOLnB extracts strongly inhibited hydroxyl radicals with IC_{50} values **(Figure 3)**.

Reducing power Assay

In this assay, Fe (III) reduction is frequently used as a significant indicator of electron donating activity, which is an important mechanism of phenolic antioxidant action by donating a hydrogen atom to break the radical chain. MOLC, MOLEA, and MOLnB (50-450 µg/ml) reduced power increased with concentration. When compared to MOLC, MOLEA and MOLnB demonstrated more effective reductive ability. When compared to the other extracts, MOLEA and MOLnB demonstrated the highest activity in a dose-dependent manner **(Figure 4)**.

Hepatoprotective Activity

Acute toxicity (LD50) studies

Acute toxicity studies were performed according to the OECD guidelines (up and down method). No mortality was observed for MOLEA and MOLnB up to 2000 mg/kg body weight. Therefore, doses of 50 mg/kg and 100 mg/kg body weight were chosen to evaluate the hepatoprotective activity of MOLEA and MOLnB extracts, respectively.

Effect of MOLEA and MOLnB on serum biochemical parameters in CCl_4 induced hepatotoxicity in rats

Administration of CCl_4 (2 ml/kg i.p.) significantly increased the levels of ALT, AST, ALP total and direct bilirubin, TG and total protein as compared to normal control. MOLEA (50 mg/kg & 100 mg/kg) and MOLnB (50 mg/kg & 100 mg/kg) reversed the elevated biochemical parameters as compared to CCl_4 treated group ($p<0.001$) **(Table 1)**.

Effect of MOLEA and MOLnB on endogenous antioxidant enzyme

The treatment of rats with CCl_4 group showed decreased SOD and CAT levels and an increase in LPO level in terms of TBARS as compared to control. MOLEA (100 mg/kg) and MOLnB (50 mg/kg) increased the SOD levels showing significant values. MOLnB (50 mg/kg & 100 mg/kg) increased the levels of CAT significantly. MOLnB (50 mg/kg) significantly inhibited the *in-vivo* lipid peroxidation **(Table 2)**.

Histopathology of liver

In the normal liver, normal liver cell arrangement was observed. There was no inflammation in the central vein, portal tract, or normal Kupffer cells **(Figure 5A)**. Hepatocytes in the CCl_4 treated group showed extensive adipocyte degeneration,

central vein and sinusoid congestion, patchy necrosis, focal hemorrhage, ballooning, and inflammation of liver cells **(Figure 5B)**. The hepatic globular structure was found to be normal in the group treated with standard silymarin (100 mg/kg). There was some central venous and sinus congestion. Hepatocyte regeneration was also discovered **(Figure 5C)**. MOLEA (50 mg/kg) treated livers showed normal reorganization of hepatocytes surrounding the necrosis and fat vacuoles **(Figure 5D)**. MOLEA (100 mg/kg) treatment of the liver resulted in mild congestion of the central vein and sinusoids. Mild inflammation and focal haemorrhage were observed, with no evidence of liver cell degeneration or swelling **(Figure. 5E)**. MOLnB (50 mg/kg) treatment reduced hepatocyte inflammation in livers. The injured liver's slight ballooning and degeneration were restored **(Figure 5F)**. MOLnB (100 mg/kg)-treated livers also showed hepatocyte regeneration with mild inflammation and ballooning **(Figure 5G)**.

PCR sequential analysis of MOL1 using r-DNA sequential analysis

Genomic DNA isolation of the endophytic fungi was performed using PCR (polymerase chain reaction) amplification of r-DNA. Single colonies of endophytic fungi were collected from agar plates and subjected to direct colony PCR. 1% agarose gel slabs were used to run the sample. The 600 bp amplicon was excised from the gel, purified and sent for sequencing **(Figure 6)**. Endophytic fungi MOL1 was isolated from *Moringa oleifera* leaves was identified as *Rhizopus stolonifer* isolate AR1 **(Figure 7)**.

Figure 1: *Rhizopus stolonifer* AR1 culture (MOL-1)

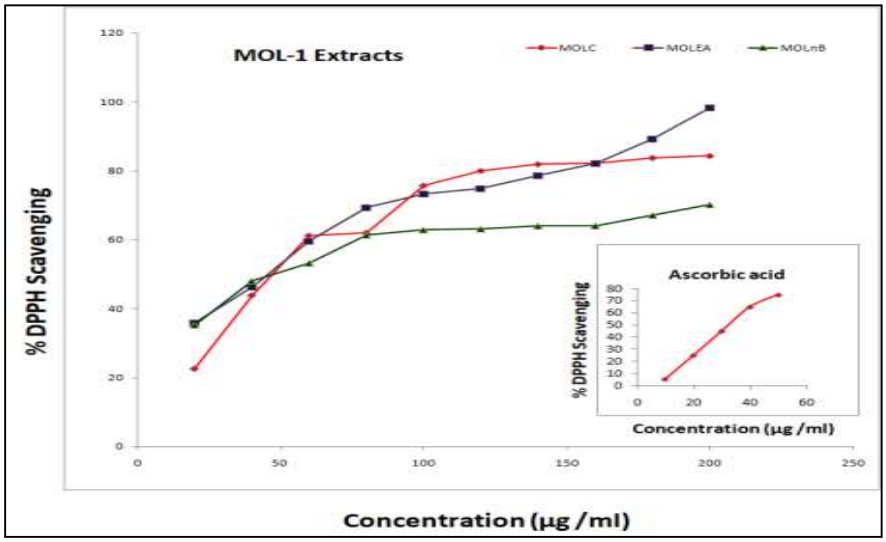

Figure 2: DPPH assay of MOLC, MOLEA and MOLnB

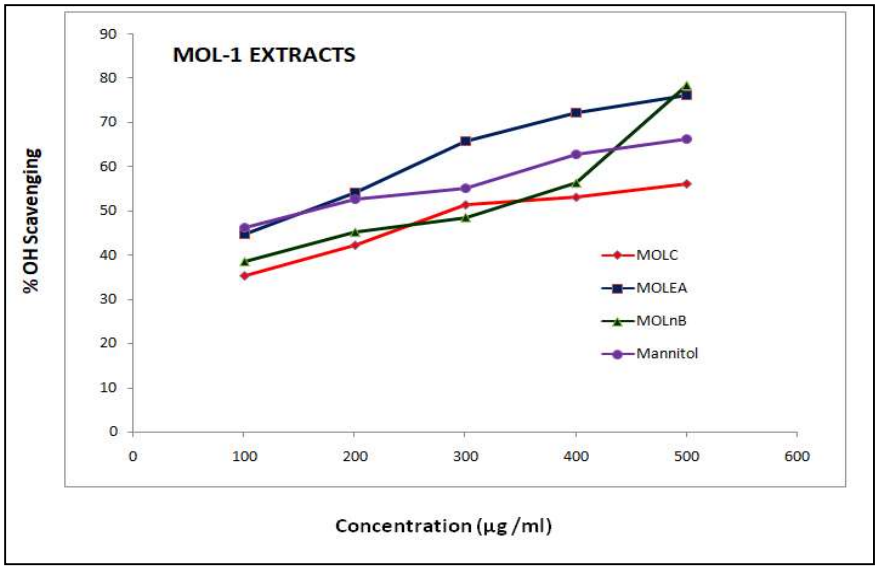

Figure 3: Hydroxyl radical assay of MOLC, MOLEA and MOLnB

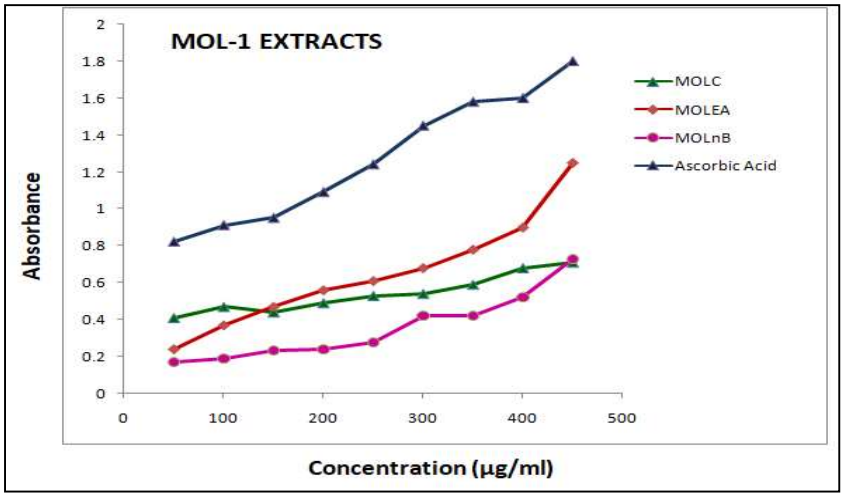

Figure 4: Reducing power Assay of MOLC, MOLEA and MOLnB

Table 1: Effect of MOLEA and MOLnB on serum biochemical parameters in CCl_4 induced hepatotoxicity in rats

Groups	SGPT (mg/dl)	SGOT (mg/dl)	SALP (mg/dl)	Total Bilirubin (mg/dl)	Direct Bilirubin (mg/dl)
Control (2 ml/kg)	229.10±0.27	236±0.67	295.1 ±6.02	0.039±0.007	0.019±0.002
CCl_4 treated (2 ml/kg)	595.5±0.13***	684±0.54**	784.2±2.82***	0.871±0.019*	0.87±0.004**
Silymarin+CCl_4 (200 mg/kg)	297.4±2.08	325±1.19	369.2±12.47	0.621±0.010	0.063±0.003
MOLEA+ CCl_4 (50 mg/k g)	275.3 ±2.51*	393±0.92*	588.7±16.30***	0.589±0.011*	0.58±0.002***
MOLEA+ CCl_4 (100 mg/kg)	282.5 ±1.69	367±1.50	595 .5±24.15*	0.431±0.010	0.59±0.002
MOLnB+ CCl_4 (50 mg/kg)	357.3±1.35***	415±10*	570.0±16.52**	0.573±0.014	0.61±0.002
MOLnB+ CCl_4 (100 mg/kg)	453.1 ±2.41	321 ±1.83	612.3±2.00*	0.581±0.005	0.61±0.003

The data were expressed as Mean ± S.E.M for six rats in each group. Statistical comparison were performed by one way ANOVA followed by Tukey's post test, *P<0.05, **P<0.01, ***P<0.0001 compared to normal control treated group

Table 2: Effect of MOLnB and MOLEA fractions on LPO, SOD and CAT levels in CCl_4 induced rats

Groups	Dose (mg/kg)	LPO level (µmol/mg protein)	SOD level (µmol/mg protein)	CAT level (µmol/mg protein)
Control	2 ml/kg	0.03±0.003	0.05±0.009	0.22±0.008
CCl_4 induced	2 ml/kg	0.17±0.005***	0.01±0.02***	0.17±0.006***
Silymarin	200 mg/kg	0.04±0.0006**	0.02±0.22***	0.23±0.002*
MOLEA 50	50 mg/kg	0.13±0.004*	0.03±0.015**	0.19±0.01**
MOLEA 100	100 mg/kg	0.11±0.001***	0.02±0.02***	0.18±0.006**
MOLnB50	50 mg/kg	0.14±0.001*	0.03±0.014**	0.22±0.07***
MOLnB100	100 mg/kg	0.16±0.005*	0.04±0.02**	0.21±0.01*

The data were expressed as Mean ± S.E.M for six rats in each group. Statistical comparison were performed by one way ANOVA followed by Tukey's post test, *P<0.05, **P<0.01, ***P<0.0001 compared to normal control treated group

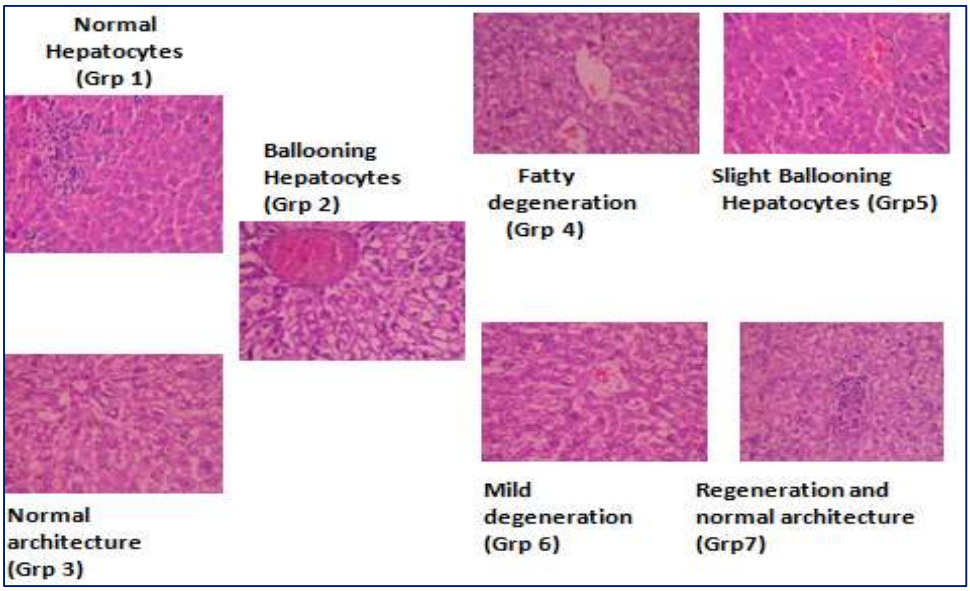

Figure 5: Group 1-Normal rat liver; Group 2-CCl_4 induced rat liver; Group 3-Silymarin treated rat liver; Group 4-MOLEA (50mg/kg); Group 5-MOLEA (100mg/kg); Group 6-MOLnB (50mg/kg); Group 7- MOLnB (100mg/kg)

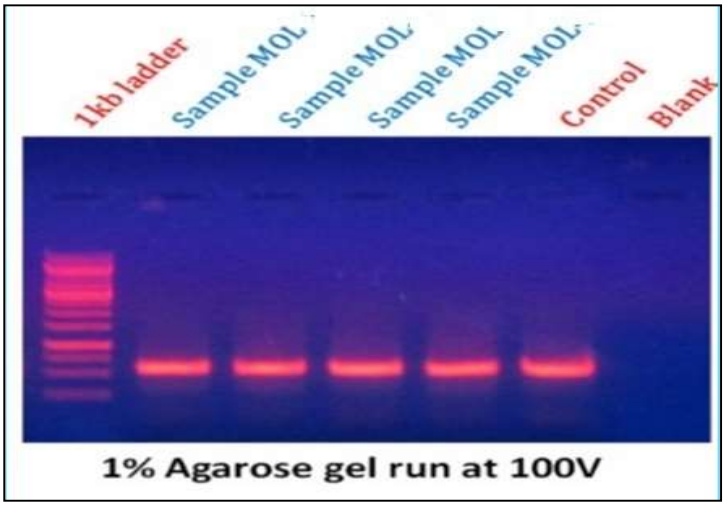

Figure 6: Genomic DNA extracted from MOL-1 run on 1% Agarose gel electrophoresis and visualized with propidium iodide

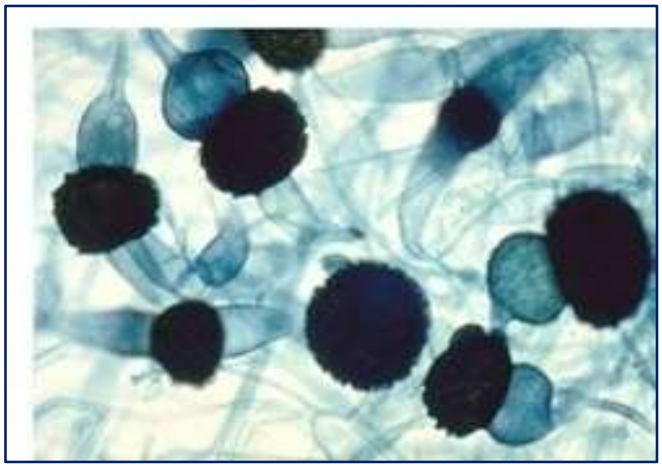

Figure 7: Endophytic fungi *Rhizopus stolonifer* isolate AR1

DISCUSSION:

The primary goal of this study was to assess the antioxidant and hepatoprotective activity of *Moringa oleifera* Lam. endophytic fractions ethyl acetate (MOLEA) and n-butanol (MOLnB) against CCl_4 induced hepatotoxicity in albino Wister rats. PCR analysis was used to isolate and characterize one endophytic fungus. Endophytic fungi were identified as *Rhizopus stolonifer* isolate AR1 using PCR analysis. The fungi identified as *Rhizopus stolonifer* by PCR have a unique, valuable ability that is used for restorative purposes.

White bread mould is another name for *Rhizopus stolonifer*. It is a zygomycota member and the most important species in the *Rhizopus* genus. It is one of the most common fungi on the planet, with a global distribution that is dominated by tropical and subtropical region **[32, 33, 34]**. *Rhizopus stolonifer* can modify steroids like progesterone to treat hormone imbalances. The microbial biotransformation of steroids

produced a number of novel metabolites with diverse activities. The metabolites produced by *Rhizopus stolonifer* from pregnenolone acetate were tested against tyrosinase and cholinesterase and showed significant inhibitory activities **[35]**.

Rhizopus stolonifer produces steroids due to a steroid hydroxylating protein that confuses and restricts localities on its plasma layer36. A delta 6-fatty acid desaturase gene from *Rhizopus stolonifer* strain YF6, which can accumulate high levels of gamma-linolenic acid, was cloned and expressed **[37]**. The ethyl acetate extract of *Rhizopus stolonifer* has potent cytotoxic activity against brine shrimp and serves as a lead compound for anticancer compounds. Its antifungal activity also suggests that it has the potential to be developed into an antifungal product **[38]**. MOLEA and MOLnB fractions were found to contain carbohydrates, alkaloids, tannins, and flavonoids in preliminary phytochemical analysis. In addition, the endophytic fractions of MOLEA and MOLnB were tested for *in vitro* antioxidant activity, including DPPH scavenging, hydroxyl free radical, and reducing activity. MOLEA and MOLnB demonstrated DPPH scavenging activity with IC_{50} value of 59.43 µg/mL and 68.91 µg/mL, respectively, while ascorbic acid had an IC_{50} value of 30.17 µg/mL.

The role of antioxidants is complex and is influenced by numerous factors. A more complex approach towards is to use diets rich in antioxidants, which improve health and can slow or protect living organisms from damage. ROS such as O_2 and OH, byproducts of normal metabolism are found in all types of organisms 39. In terms of antioxidant activity, oxidative stress is defined as an imbalance between cellular production of reactive oxygen species (ROS) and cellular capacity to scavenge those (OS). OS has been suggested as a possible factor in the aetiology of a variety of disorders **[40]**. The activity of free radical scavenging models such as DPPH, hydroxyl radical, and reducing power was investigated.

DPPH is a synthetic, stable radical that does not degrade in water, methanol, or ethanol. At 517 nm, the DPPH can accept an electron or hydrogen from antioxidant molecules in order to form a stable diamagnetic molecule **[29]**. The reactions of MOLEA and MOLnB with DPPH radicals demonstrated significant scavenging activity.

The Fenton reactions, fatty acid oxidation, and xenobiotic detoxification generate hydroxyl radicals in the mitochondria and endoplasmic reticulum. Endophytic extracts demonstrated dose-

dependent hydroxyl radical scavenging activity. This could be due to the iron ions chelating and avoiding deoxyribose degradation, thereby stopping complexation with the deoxyribose and rendering it inactive in a Fenton reaction. When compared to EDTA **[41]**, MOLEA and MOLnB reduced the metal concentration in the Fenton reaction, demonstrating the chelating effect on ferrous ions. With increasing concentration, the reducing power of MOLEA and MOLnB (50-450 µg/ml) and ascorbic acid gradually increased. This action could be attributed to mono and dihydroxyl substitutions in the aromatic ring, which have strong hydrogen donating properties.

The endophytic fractions MOLEA and MOLnB were tested for acute toxicity using OECD guidelines (up and down method). *Moringa oleifera* Lam leaves did not cause toxicity or behavioural changes in mice at doses of up to 2000 mg/kg, so doses of 50 and 100 mg/kg were chosen for hepatoprotective effect and activity. Serum SGOT, SGPT, SALP, total bilirubin, direct bilirubin, and total protein activities were determined.

CCl_4 induced liver cell damage begins with the endoplasmic reticulum's mixed capacity oxidase assembly converting CCl_4 to trichloromethyl free radicals. It is proposed that the optional system connects CCl_4 digestion to the widespread troubling effect on hepatocyte function. Minor components may include the ageing of toxins caused by CCl_4 digestion or peroxidative degeneration of layered lipids. The free radicals produced caused self-oxidation of the unsaturated fats found in the cytoplasmic film's phospholipids, resulting in morphological changes in the cell layer. Furthermore, the tide of extracellular Ca^{++} particles within the cell is substantial. Hepatocellular toxicity causes a large amount of SGOT and SGPT to be released into the bloodstream from liver tissue. Because liver SGPT accounts for 90% of all compounds present in the body, it is considered the superior enzyme in liver damage. SALP activities are associated with hepatocyte activity; the increase in their effect is due to a prolonged combination in the vicinity of enlarged bile mass.

The decrease in SGOT and SGPT values to normal indicates that the plasma layer has been adjusted and that the CCl_4 induced damage to liver tissues has been repaired **[42]**. The effect of extreme metabolites on liver detoxification is due to catalase, superoxide dismutase (SOD), and glutathione, as well as decreased protein movement and compound inactivation during the synergist cycle. The current study

found that MOLEA and MOLnB (50 and 100 mg/kg) increased depleted enzyme levels caused by CCl_4 toxicity, protecting hepatocyte structural integrity and promoting liver cell regeneration. Both fractions had a significant effect on the performance of hepatic enzymes involved in combating reactive oxygen species (ROS).

The activities of various enzymes as an indicator of liver damage and pathological disorders caused by CCl_4 demonstrated the hepatoprotective effect of *Moringa oleifera* Lam associated fungal fractions. The endophytic fractions of *Moringa oleifera* Lam. leaves protect the liver from oxidative damage and could be used as a potent protector in CCl_4 hepatotoxicity damage, according to the findings of this study. The presence of antioxidant phytochemicals may be primarily responsible for the activity.

Furthermore, the histopathological examination of the liver revealed evidence of the effects of the investigated components on acute CCl_4 induced liver injury, corroborating the biochemical analysis. Histology revealed that CCl_4 administration resulted in severe oxidative liver damage characterized by severe necrosis, inflammation, hepatocellular degeneration, cytoplasmic vacuolation, and loss of cellular boundaries, which is consistent with previous studies for liver injury **[43]**.

CONCLUSION:

MOLEA and MOLnB (50 and 100 mg/kg) of the *Moringa oleifera* leaf endophytic fungus *Rhizopus stolonifer* AR1 exhibited antioxidant and hepatoprotective activity in CCl_4 induced hepatotoxicity.

CONFLICT OF INTEREST:

The authors have no conflicts of interest regarding this investigation**.**

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