IN-VITRO ANTIOXIDANT AND ANTICANCER ACTIVITY OF *MADHUCA LONGIFOLIA*

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Abstract

In the current study, the antioxidant properties of *Madhuca longifolia* was effectively explored utilizing *DPPH radical scavenging, Nitric oxide radical scavenging, Superoxide anion radical* scavenging, *Total antioxidant activity* by $ABTS^{\bullet+}$ radical scavenging and *lipid peroxidation inhibitor* assays and compared with the standards while anticancer property was evaluated by MTT assay using MCF-7 Cell line.

Percentage growth inhibition by MTT assay using MCF-7 cell line was found to be 61.66±1.34% by MLHA compared to 83.03±1.0% by standard drug Methotrexate.

1. Introduction

Herbal plants play a significant job in moderating human wellbeing and improving the value of human life for a large number of years [1]. Since most recent couple of years, an immense advancement in the region of herbal medication has been experienced and these natural medications are achieving prominence in developing as well as in developed countries, as they have lesser side effects. These herbal plants are an incredible wellspring of secondary metabolites having important biological actions [2-4].

Free radicals are generating naturally in the body and express an important part in numerous normal cellular activities. An increase in concentrations of free radicals can be injurious to the body and damage all major mechanisms of cells including DNA, proteins, and cell membranes which may likewise assumes that it may role in the advancement of malignant and other health conditions. Antioxidants are substances that interact and neutralize free radicals, therefore keeping away from them to influencing harm [5,6].

Madhuca longifolia well- known as the Butter nut tree belongs to *Sapotaceae* family. It is a medium to large sized deciduous tree. The flowers have analgesic and diuretic property whereas bark is used to treat rheumatism, diabetes and bronchitis [7]. leaves of *Madhuca longifolia* are expectorant and also used for Cushing‟s disease, gastropathy, dipsia, dermatopathy and hemorrhoids [8,9]. This work aimed to study the antioxidant and anticancer activity of different extracts of leaves of *Madhuca longifolia.*

2. Materials and methods

2.1 Collection and Authentification of the Plant

The Leaves of *Madhuca longifolia* was bring from botanical garden of Ashtang Ayurvedic College, Indore and authenticated by Dr. Shakun Mishra, Head of the Department (Botany), Shree S.N Govt. P.G. College, Khandwa. A Voucher specimen of plant has been preserved for further references.

2.2 Preparation of extracts of *Madhuca longifolia leaves.*

The powdered plant material about 1000 gm of plant leaves were successively extracted using petroleum ether, ethanol, hydroalcoholic(1:1) and distilled water in Soxhlet apparatus. After about forty siphons of each solvent extraction step, the materials were concentrated by evaporation [10,11].

2.3 Drugs and Chemicals

DPPH, sodium nitroprusside, sulphanilamide, naphthylethylene diamine dihydrochloride, Curcumin, nitroblue tetrazolium, NADH, phenazine methosulphate, Catechin, ferric chloride, L-ascorbic acid, trichloroacetic acid, thiobarbituric acid, butylated hydroxy anisole, TROLOX, ABTS, potassium persulfate obtained from Sigma Chemical Co. Ltd, USA. All chemicals and solvents were of reagent grade.

2.4 Preliminary phytochemical screening

Different extracts of *Madhuca longifolia* was exposed to qualitative tests for the detection of various active components viz. flavonoids, alkaloid, glycoside, amino acids, carbohydrate, fixed oil, tannins, phytosterols, gum and mucilage etcby means of chemical tests.

2.5 Free Radical Scavenging and antioxidant activity

In the present study, the antiradical and antioxidant activities of the different extracts in five *in-vitro* models, including *DPPH radical scavenging, Nitric oxide radical scavenging, Superoxide anion radical* scavenging, *total antioxidant activity* by $ABTS^{\bullet+}$ radical scavenging and *lipid peroxidation inhibitor* assays were inspected.

2.5.1 DPPH radical scavenging activity

DPPH *i.e.* 2,2-diphenyl-1-picrylhydrazyl, an organic chemical compound. In this assay, violet color DPPH solution is reduced to yellow colored product by the addition of the extract in a concentration dependent manner which were detect at 517nm [12].

Add 0.1mM solution of DPPH reagent in methanol and measure the absorbance. Take 1ml of this solution and add 3ml methanolic solution of different extract in concentration of 10, 20, 30, 40, 50 and 100 µg and incubate for 30 min at 517nm and measure the absorbance. Ascorbic acid was taken as reference drug. Perform the experiment in triplicate and the results averaged.

Percentage scavenging of DPPH radical was calculated using the formula

$$\% \text{ Scavenging of DPPH} = \frac{[\text{Absorbance of Control- Absorbance of Test}] \times 100}{[\text{Absorbance of Control}]}$$

2.5.2 Nitric oxide radical scavenging effect:

Scavenger of nitric oxide compete with oxygen leading to reduced production of nitric oxide in body [13,14]. Preparation of reaction mixture (3ml), by adding sodium nitroprusside (10 mM) in phosphate buffered saline (PBS) and add the extracts in different concentrations (10, 20, 30, 40, 50 and 100 µg)**,** incubate mixtures at

25°C for 150 min. At an interval of every 30 min, 0.5 mL of the incubated extracts was removed and 0.5 mL of Griess reagent (1% sulphanilamide, 0.1% naphthylethylene diamine dihydrochloride in 2% H_3PO_4) was added. The chromophore formed was measured at an absorbance of 546 nm. Performed this experiment in triplicate and averaged. The percentage inhibition of nitric oxide generated was measured by comparing the absorbance values of control and test. Curcumin was used as a reference compound.

2.5.3 Superoxide anion radical scavenging effect:

Add1 mL of nitroblue tetrazolium (NBT) solution (156 µM NBT in 100 mM phosphate buffer, pH 7.4), 1 mL of NADH solution (reduced form of β-nicotinamide adenine dinucleotide) (468 µM in 100 mM phosphate buffer, pH 7.4) and 0.1 mL of extracts in different concentration (10, 20, 30, 40, 50 and 100 µg) in distilled water. By additing of 100 µl of phenazine methosulphate (PMS) solution (60 µM PMS in 100 mM phosphate buffer, pH 7.4) reaction was started. Incubated reaction mixture at 25^0C for 5 min and absorbance was measured at 560 nm against blank samples. Decreased absorbance of the reaction mixture denote increased scavenging acitivity. Perform experiment in triplicate and then averaged. Catechin used as reference compound. The percentage of inhibition was determined by comparing the results of control and test samples [15].

2.5.4 Lipid peroxidation inhibitory activity

300mg of egg lecithin with 30ml of phosphate buffer, pH 7.4 in an ultrasonic sonicator for 30 min to formulate liposome. Add plants extracts and standard (ascorbic acid) of different concentrations (10, 20, 30, 40 50, and 100 µg/mL) and incubate for 10min. Add ferric chloride (0.5ml, 400 mM) and L-ascorbic acid (0.5ml, 400 mM) to induce Lipid peroxidation and incubated for 1 h at 37^0C. By adding hydrochloric acid (2 mL, 0.25 N) containing trichloroacetic acid (TCA, 150 mg/ mL), thiobarbituric acid (TBA, 3.75 mg/mL) and butylated hydroxy anisole (BHA, 0.50 mg/mL) reaction was stopped. Heat the reaction mixture at 80^0C for 20 min, cooled, centrifuged for 10 min and the absorbance of the supernatant liquid was measured at 532 nm [16-18]. Experiments were performed in triplicate and averaged, the % inhibition at varying concentration was calculated by the following formula

$$\% \text{ Inhibition} = [1-(Vt/Vc)] \times 100$$

Where, Vt = mean absorption of test, Vc= mean absorption of control

The IC_{50} value was derived from the % inhibition at different concentration.

2.5.5 Total antioxidant activity

ABTS●+ [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] is a radical cation generated directly in stable form through the reaction between ABTS and potassium persulfate. Plant extract containing

antioxidants reduce ABTS after reaction, and thus the extent of radical's inhibition is determined and calculated relatively to standard TROLOX.

Make a 7 mM stock solution by dissolve ABTS in water. Add 2.45 mM potassium persulfate to this to ensure generation of $ABTS^{\bullet+}$ radical cation. Allow mixture to stand up to 12–16 h in the dark at room temperature, then measure the absorbance, after stable absorbance, then it was ready to use. The ABTS●+ solution was diluted with water and equilibrated to 0.70 (± 0.02) at 30°C. Add 10 µL of plant extracts and 0-15 µL of standard TROLOX separately to 1 ml ABTS●+ solution and analyze the absorbance at 734 nm after 6 min, perform this experiment at least three times [19-21].

For calculation of TEAC, the gradient of the plot for the sample was divided by the gradient of the plot for TROLOX and scavenging activity of the samples calculated by the formula-

$$\mathbf{S\% = [(A_{control} - A_{sample})/A_{control})]\times 100,}$$

Where $A_{control}$ is the absorbance of the blank control ($ABTS^{\bullet+}$ solution without test sample) and A_{sample} is the absorbance of the test sample.

2.6 *In vitro* cytotoxicity activity by MTT assay method

The plants extracts were subjected for *in vitro* cytotoxicity activity by MTT assay method using MCF 7 cell line. MTT assay is based on the supposition that dead cells or their products do not reduce tetrazolium. The assay relies on both the mitochondrial activity per cell and number of cells present. The assay is based on the principle that the mitochondrial enzyme succinate dehydrogenase present in living cells cleaves 3-(4, 5 dimethyl thiazole-2yl)-2, 5- diphenyl tetrazolium bromide (MTT) into a blue formazan derivative. The amount of cells was found to be proportional to the extent of formazan production by the cells used [22].

Cells were seeded in a 96-well flat-bottom plate (5000 cells/well) and permitted to adhere for 24h at 370 C with 5% CO2 atmosphere. Different drug concentration was added and incubated further for 48hrs. Before 4h of the completion of incubation, 20µl of MTT (5mg/ml) was added. Dead cell percentage was determined using an ELISA plate reader set to record absorbance at 570nm. The percentage growth inhibition was calculated using the formula given below[23]

% growth inhibition = 100 X (Control OD-Sample OD/Control OD)

Sample OD=Absorbance of treated cells (Plant Extracts and Standard Drug)

Control OD=Absorbance of control (untreated)

3. Results and Discussion

3.1 Phytochemical Screening

The extraction was performed by using different solvents ranging from polar to nonpolar for eg. Distilled water, hydroethanolic (1:1), ethanol, and petroleum ether. The abbreviations used for the extracts along with the extractive values are reported in the *Table I*. The phytochemical screening of all extracts showed positive

reaction for the flavonoids, phenols, saponin, alkaloids, triterpanoids and tannins. The results of phytochemical analysis depicted in the *Table II.*

Table I: Abbreviations for extracts and Extractive values of extracts

S. No.	Plant	Extract	Abbreviation used	Extractive values
1.	*Madhuca longifolia*	Petroleum ether	MLPE	47.33
		Ethanol	MLET	42.15
		Hydroalcoholic	MLHA	37.12
		Distilled water	MLDW	38.16

Table II: Phytochemical analysis of leaves extracts of *Madhuca longifolia* by using different solvents.

S. No.	Phytochemicals	MLPE	MLET	MLHA	MLDW
1	Steroids	-	-	-	-
2	Glycosides	-	-	-	-
3	Fatty acids	+	-	-	-
4	Anthraquinone glycoside	-	-	-	-
5	Alkaloids	+++	-	+	+
6	Phenols	+++	+	+	+
7	Flavanoids	-	+	++	+
8	Saponins	-	+	++	+
9	Tannins	-	-	+	+
10	Triterpenoids	-	+	+	+

Where symbol (+++) indicates presence in high concentration, symbol (++) indicates presence in moderate concentration, symbol (+) indicates presence in trace concentration and symbol (–) indicates absence of the respective phytochemical.

3.2 DPPH free radical Scavenging assay

The antioxidant reacts with stable free radical DPPH and converts it to 1,1-diphenyl-2-picryl hydrazine. The ability to scavenge the free radical, DPPH was measured at an absorbance of 516 nm. So the DPPH and its % inhibition of petroleum ether, ethanol, hydroalcoholic and distilled water extract reported in table III and *Figure I* . Ascorbic acid has taken as reference standard which showed 67.45 ± 3.467 % inhibitions. Among these results the extracts MLHA have more significantly potent activity than other extracts and showed 44.52±0.23% inhibition respectively.

Table III. DPPH radical scavenging activity by *M. longifolia extracts*

Sample	10 µg/mL	20µg/mL	30 µg/mL	40 µg/mL	50 µg/mL	100 µg/mL	IC50
Ascorbic acid	17.64±0.03	30.41±0.02	43.53±0.13	56.80±0.04	68.43±0.02	95.25±0.03	39.29
MLPE	3.45±0.04	8.00±0.27	14.55±0.04	19.47±0.37	22.89±0.27	31.06±0.47	153.92
MLET	10.55±0.14	17.64±0.25	22.56±0.05	27.61±0.13	31.20±0.44	41.41±0.34	118.32
MLHA	12.11±0.67	17.57±0.09	22.97±0.36	27.58±0.36	31.27±0.08	44.52±0.23	110.22

MLDW	6.07±0.07	11.38±0.21	13.88±.006	18.44±0.31	23.44±0.07	35.42±0.25	141.16

All readings are mean ± SD, n = 3, IC50 value reported as Conc. ± SEM *P < 0.05, **P < 0.01

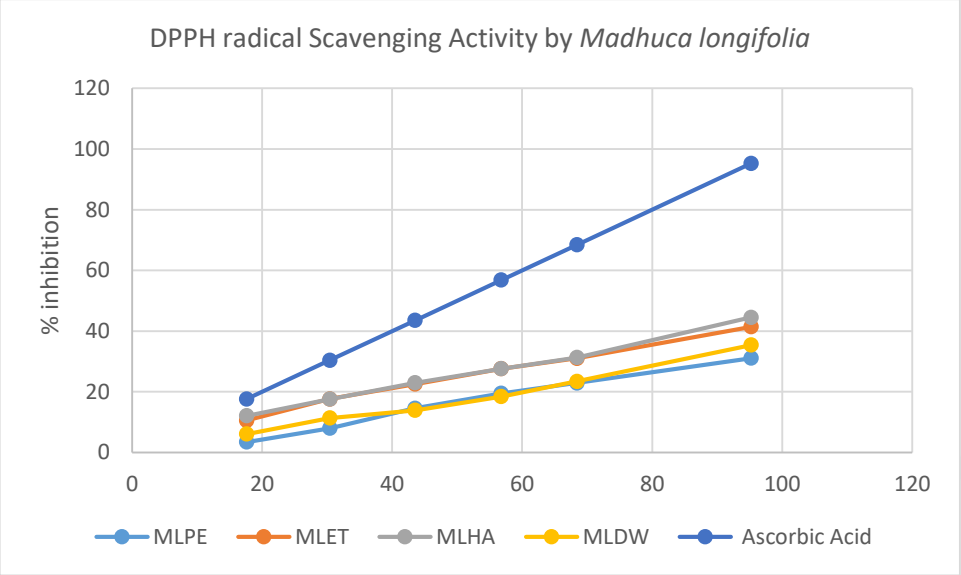

Figure I. DPPH radical scavenging activity by plants extract and ascorbic acid.

3.3 Nitrous oxide radical scavenging activity

Nitric oxide (NO), a vital chemical mediator generated by endothelial cells, macrophages, neurons, etc., involved in the regulation of various physiological procedures. Excess concentration of NO is correlated with a number of diseases. Oxygen reacts with the excess nitric oxide to generate nitrite and peroxynitrite anions, which behave as free radicals. In the present study, the tested compounds compete with oxygen to react with nitric oxide and thus restrains formation of the anions. *Table IV* and *Figure II* demonstrates the percentage inhibition of nitric oxide production by varying concentrations of tested compounds. Curcumin has been use as a standard. The potent % inhibition value of extracts was found to be 52.40±0.10% exhibited by MLHA compared to standard curcumin which produce 91.42±0.22% inhibition.

Table IV. Nitrous oxide radical scavenging activity by *M. longifolia* extracts

Sample	10 µg/mL	20µg/mL	30 µg/mL	40 µg/mL	50 µg/mL	100 µg/mL	IC50
Curcumin	16.12±0.24	29.86±0.14	38.42±0.02	48.76±0.04	61.35±0.32	91.42±0.22	44.57
MLPE	6.88±0.02	9.96±0.17	11.39±0.38	15.37±0.28	18.80±0.28	31.69±0.23	165.11
MLET	12.75±0.01	16.74±0.21	21.00±0.11	25.50±0.10	33.12±0.06	50.20±0.01	97.09
MLHA	7.13±0.18	15.78±0.05	22.92±0.24	30.99±0.30	36.83±0.07	52.40±0.10	87.48
MLDW	4.29±0.22	11.02±0.01	15.98±0.13	19.64±0.15	23.72±0.05	36.47±0.21	134.11

All readings are mean ± SD, n = 3, IC50 value reported as Conc. ± SEM *P < 0.05, **P < 0.01

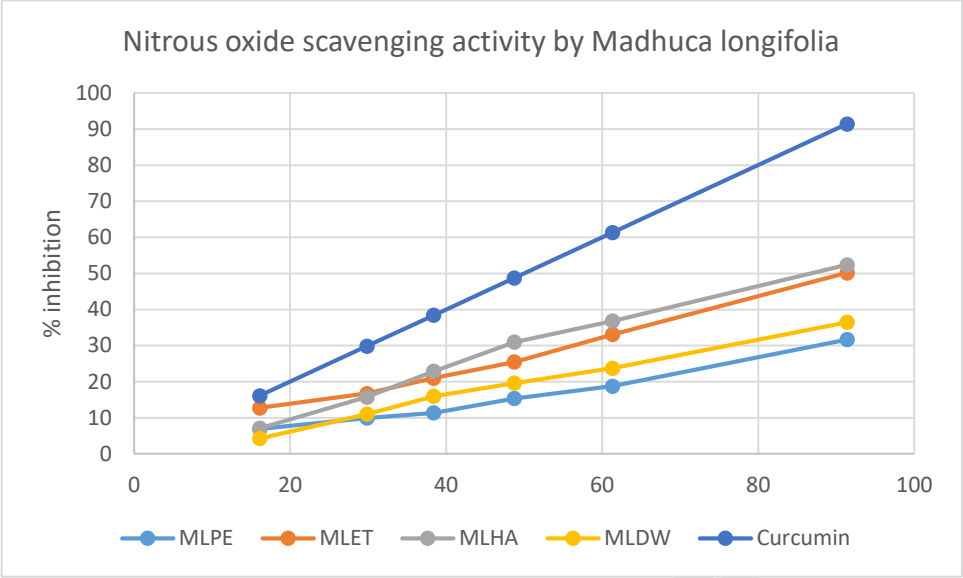

Figure II. Nitrous oxide radical scavenging activity by plants extract and standard Curcumin.

3.4 Superoxide anion radical scavenging effect

Table V and Figure III shows the superoxide scavenging effect of each plants extract and catechin on the PMS/NADH-NBT system. The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. The potent % inhibition value of extracts was found to be 59.78±0.14% exhibited by MLHA compared to standard catechin which produce 93.14+0.34% inhibition.

Table V. Superoxide radical scavenging activity by *M. longifolia* extracts

Sample	10 µg/mL	20µg/mL	30 µg/mL	40 µg/mL	50 µg/mL	100 µg/mL	IC50
Catechin	31.51± 0.80	49.28±0.52	65.22±0.41	76.40±1.32	89.23±0.28	93.14±0.34	39.53
MLPE	5.68±0.16	9.86±0.26	20.09±0.48	25.27±0.67	32.82±0.62	43.78±0.82	105.48
MLET	4.29±0.54	9.05±0.13	22.72±0.58	29.74±0.51	37.57±0.19	51.68±0.82	87.44
MLHA	12.71±0.26	23.36±0.32	31.77±0.15	39.14±0.08	46.35±0.06	59.78±0.14	70.77
MLDW	9.79±0.08	20.04±0.07	28.35±0.03	32.29±0.12	41.21±0.11	44.58±0.02	99.41

All readings are mean ± SD, n = 3, IC50 value reported as Conc. ± SEM *P < 0.05, **P < 0.01

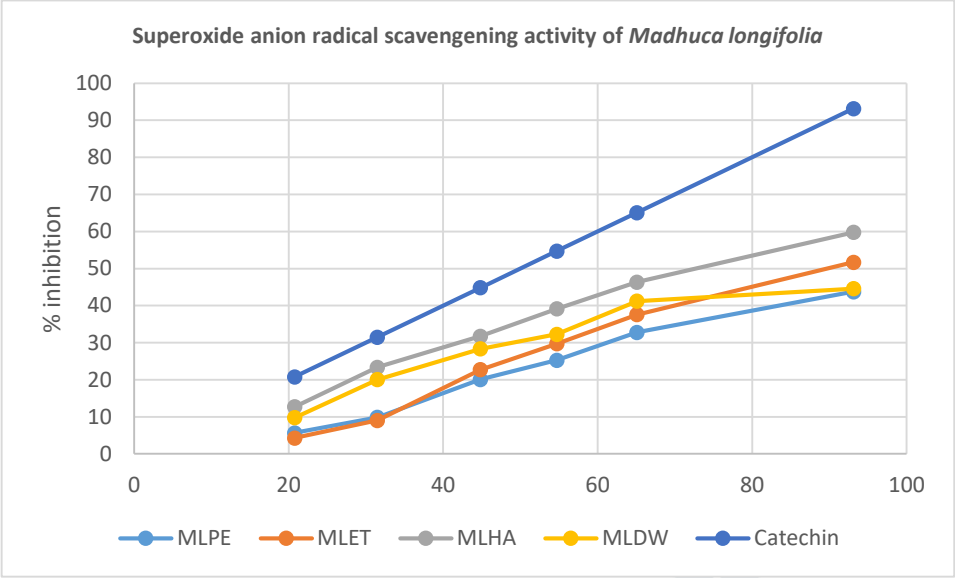

Figure III. Super oxide anion radical scavenging activity by Catechin and plants extracts.

3.5. Lipid peroxidation inhibitory activity

Lipid peroxidation precursors are reactive oxygen species which are formed by exogenous chemical factors and endogenous metabolic processes in the human body or in food systems. We tested the scavenging activity of each extract along with standard drug Ascorbic acid at different concentration shown in *Table VI* and *Figure IV*. Inhibition of radical were found to be 37.51±0.06% exhibited by MLHA compared to standard Ascorbic acid which produce 99.83+0.047% inhibition.

Table VI. Lipid peroxidation inhibitory activity by *M. longifolia* extracts

Sample	10 µg/mL	20µg/mL	30 µg/mL	40 µg/mL	50 µg/mL	100 µg/mL	IC50
Ascorbic Acid	9.04± 0.30	21.47±0.03	31.64±0.43	40.60±0.36	53.26±0.36	99.83±0.047	49.03
MLPE	3.62±0.11	8.65±0.38	12.93±0.27	19.16±0.34	24.02±0.55	31.68±0.55	149.99
MLET	8.31±0.058	13.72±0.55	20.02±0.13	26.70±0.17	28.75±0.55	34.67±0.76	141.56
MLHA	8.30±0.069	16.71±0.026	23.48±0.58	26.95±0.045	30.31±0.60	37.51±0.06	130.32
MLDW	6.76±0.045	10.70±0.023	15.35±0.12	20.75±0.17	24.40±0.13	32.75±0.16	151.93

All readings are mean ± SD, n = 3, IC50 value reported as Conc. ± SEM *P < 0.05, **P < 0.01

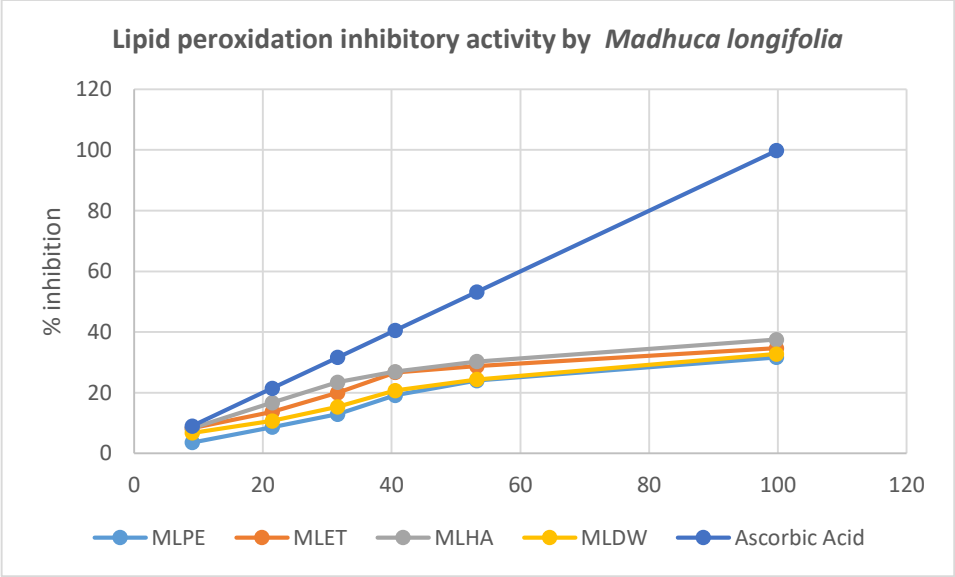

Figure IV. Lipid peroxidation activity by plants extract and Ascorbic acid

3.6 Total antioxidant activity

The antioxidant activity was measured by the inhibitory quantity of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•$^{+}$) radical cation was compared to 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (TROLOX). *Table VII* and *Figure V* have shown the comparative analysis of result. Inhibition of radical were found to be 44.52±0.23% exhibited by MLET compared to standard TROLOX which produce 97.09±0.29 % inhibition.

Table VII. Total antioxidant activity by *M. longifolia* extracts

Sample	10 µg/mL	20µg/mL	30 µg/mL	40 µg/mL	50 µg/mL	100 µg/mL	IC50
TROLOX	25.51±0.54	34.73±0.43	44.57±0.71	53.87±0.43	62.76±0.79	97.09±0.29	37.76
MLPE	5.37±0.02	10.66±0.32	14.91±0.64	22.76±0.44	28.51±0.25	36.42±0.55	129.51
MLET	9.32±0.16	17.73±0.18	25.43±0.16	32.27±0.51	37.48±0.56	44.52±0.23	101.74
MLHA	6.24±0.09	13.45±0.38	18.84±0.56	26.86±0.07	33.73±0.58	42.09±0.13	109.91
MLDW	9.35±0.04	13.75±0.17	18.73±0.05	24.02±0.03	27.15±0.19	36.11±0.44	139.45

All readings are mean ± SD, n = 3, IC50 value reported as Conc. ± SEM *P < 0.05, **P < 0.01

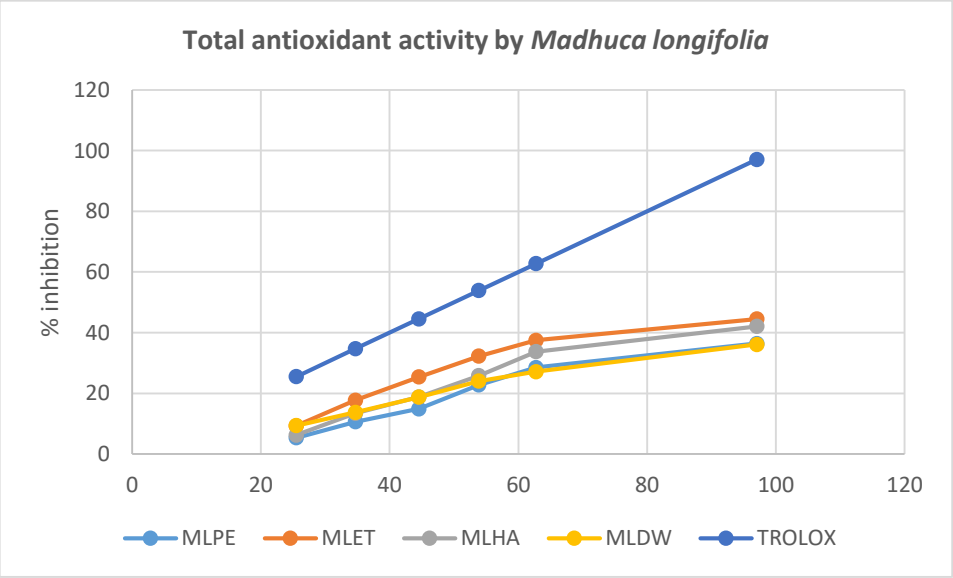

Figure V. Total antioxidant activity by plants extract and TROLOX.

3.7 *In-vitro* anticancer activity by MTT Assay on MCF-7 Human Breast cancer cell line

The effect of different plants extract on the growth of MCF-7 cell line was investigated and compared with standard drug Methotrexate by the MTT assay. The maximum percentage inhibition value obtained at 500µg concentration were 61.66±1.34 % by, MLHA compared with 83.03±1.0% by standard drug. *Table VIII* and *Figure VI* shows results of MTT assay.

Table VIII. MTT assay by *M. longifolia* extracts

Concentration (µg/ml)	% growth Inhibition				
	MLPE	MLET	MLHA	MLDW	MXT
500	37.57±0.70	57.17±10.3	61.66±1.34	32.48±0.90	83.03±1.0
166.66	25.40±0.66	43.66±0.95	51.15±0.76	23.94±0.61	69.26±1.04

55.55	23.50±0.61	41.88±0.79	49.38±0.95	22.57±0.74	65.64±0.62
18.52	22.02±0.84	39.44±0.84	46.67±1.19	21.13±0.91	63.08±0.99
6.17	19.46±0.57	37.42±0.81	43.71±0.83	19.29±1.43	59.82±0.37
2.06	18.27±0.48	35.70±1.48	41.52±0.44	16.90±1.17	57.57±0.99
0.68	16.32±0.34	33.85±1.27	39.22±0.62	14.75±1.16	55.02±0.90
0.23	14.78±0.49	31.76±1.20	37.21±0.64	12.9±1.50	52.70±1.06
0.076	13±0.79	29.80±1.43	35.08±0.69	10.75±1.40	50.45±1.72
0.025	10.56±0.68	27.60±1.43	33.19±0.80	9.08±1.06	48.43±1.51

All readings are mean ± SD, n = 3.

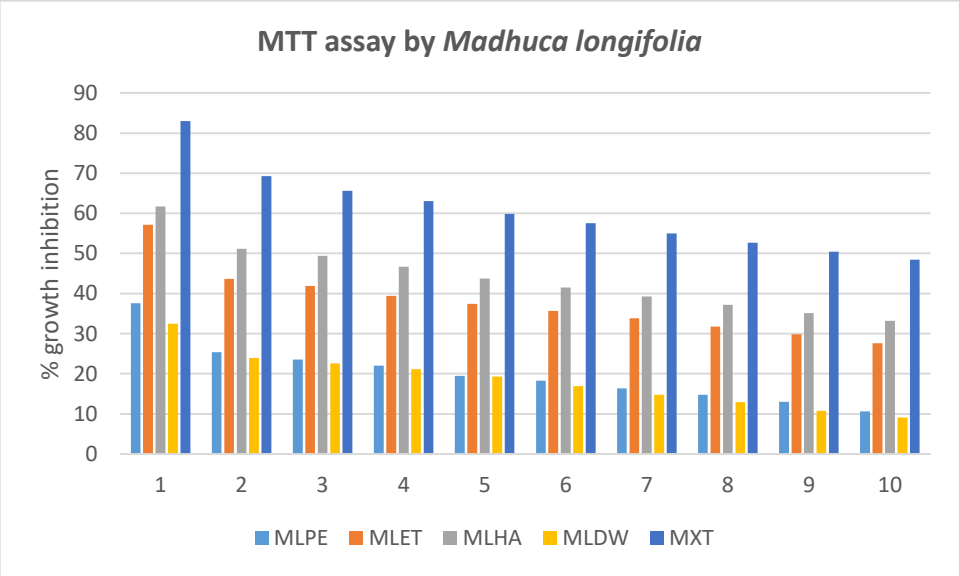

Figure VI. In- vitro cytotoxicity activity by MTT assay by Methotrexate and plants extract.

Where Series 1,2,3,4,5,6,7,8,9,10 represent concentration of 500,166.66,55.55,18.52,6.17,2.06,0.68,0.23,0.076 and 0.025µg/ml

Conclusion

It is concluding that by phytochemical evaluation it is observed that medicinal plant has highest classes of phytoconstituents and therapeutic efficacy. Amongst all the extract the hydroalcoholic extract indicate the more positive results for its both antioxidants and anticancer activity in various *in-vitro* models. Due to presence of various phytoconstituents, the plant has been utilized to treatment of different sicknesses. This research premise of its utilization in medication and create to additionally use in medicine and develop to further drugs in the treatment of malignant growth since these have great antioxidant activity and also in further drugs in pharmaceutical zone and also contains the dynamic biologically active constituents, and secondary products are important of further investigation.

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