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Phytochemical Screening and Evaluation of Antioxidant and Antiproliferative Potential of *Morus alba* (L.) Leaves Extracts against Breast Cancer Cell Lines

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Abstract

Morus alba (L.) belongs to the family Moraceae commonly known as white mulberry used in folk medicine in the remedy of dysentery, diuretic, and antidiabetic treatments. Morus species contain several bioactive compounds which attribute to their biological properties. The purpose of this study is to determine the total phenolic content, antioxidant potential and antiproliferative activity of different leaf extracts of *Morus alba* (L). The plant leaves extracts were prepared by the Soxhlet extraction method. The total phenolic content was determined by Folin–Ciocalteu method and antioxidative potential was assessed by H_2O_2 free radical scavenging assay. The antiproliferative potential was investigated by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay using breast cancer cell lines MCF-7 and MDA-MB-231. Our results showed that all the extracts exhibited significant antioxidant and antiproliferative activity dose-dependently. Amongst all, the ethanolic leaf extract possessed higher radical scavenging activity as well as potent cytotoxicity against breast cancer cells. Furthermore, the ethanolic leaf extract was subjected to analyse of its bioactive composition that might be responsible for its potent antioxidant and antiproliferative potential. The presence of different functional groups in the extract was analysed by Fourier Transform Infrared (FTIR) technique and the main bioactive compounds in the extract were identified by Liquid Chromatography-Mass Spectrometry (LC-MS). Therefore, it can be concluded by the above findings that *Morus alba* (L.) has promising antioxidant activity and potent antiproliferative activity against breast cancer cells and thus can be used as a great source of natural antioxidants in developing drugs against various diseases.

Keywords: Antiproliferative Activity, Breast Cancer, *Morus alba* (L.), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)

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1. Introduction

Researchers have reported hormonal, lifestyle and various environmental factors that may be responsible for increasing the risk of breast cancer in women. American Cancer Society's update on female breast cancer statistics in the United States reported that breast cancer is one of the leading causes of cancer-related death in women in the United States, second to lung cancer[1]. Chemotherapy and radiation therapy have been the main treatments available for its treatment in the last few decades[2]. But its prolonged usage can cause severe side effects and multidrug resistance which encourages the use of complementary and alternative treatment approaches. Studies have also shown evidence that naturally derived compounds and plant extracts exhibit anticancer activity[3]. Recent studies have demonstrated that these natural compounds from dietary sources can target several pathways related to breast cancer which can accelerate breast cancer progression and malignancies[4]. Previous findings have reported various activities of *Morus nigra* including antioxidant, anti-nociceptive, anti-inflammatory, anti-diabetic, antibacterial, cardiac depressant, vermifuge, anticancer, and maternal health[5]. An investigation has demonstrated promising hepatoprotective activity of aqueous methanolic and ethanolic extracts of *M. nigra*[6]. Another group of researchers investigated both fruits and leaves of *Morus macroura* as a potential source of bioactive compounds against Alzheimer's Disease (AD)[7]. After reviewing the literature and considering the potential action of Morus species in treating various diseases prompted to focus on identifying the bioactive composition in the *Morus alba (L.)* leaves extracts and investigating their therapeutic efficacy against breast cancer cells.

2. Material and Methods

2.1 Sample Collection

Morus alba (L.) leaves of variety (C-1360) were procured from Central Sericulture Research and Training Institute, Berhampore, West Bengal, India.

2.2 Extraction Procedure

The dried leaves of *Morus alba* (L.) were powdered and extracted using 70% ethanol, 80% methanol and distilled water for 6 hours with concomitant shaking and filtered. The extracts were then concentrated using a rotary evaporator and kept at 4°C till their use.

2.3 Chemicals, Reagents and Cell Lines

All chemicals and reagents were procured from certified suppliers and were of the highest analytical standard**,** Roswell Park Memorial Institute (RPMI) 1640, Leibovitz's L-15 Medium, Fetal Bovine Serum (FBS) and Penicillin and Streptomycin solution were purchased from Himedia Laboratories (Mumbai, India). Human Breast cancer cell lines (MCF-7 and MDA-MB-231) were obtained from NCCS (Pune, India).

2.4 Determination of Total Phenolics

The concentration of total phenolics in the extracts was determined using Folin-Ciocalteau's reagent[8]. Briefly, in 2 ml of different concentrations of gallic acid and extracts ranging from 25-250 µg/ml, 1.5 ml of 10 times diluted Folin-Ciocalteau reagent and 1.5 ml of $NaHCO_3$ was added and the mixture was kept in the dark for 60 mins. The absorbance was measured at 725 nm using a UV spectrophotometer. All results were expressed as gallic using a standard curve of gallic acid and a linear equation was used to calculate the total phenolics of the extracts.

2.5 Determination of Antioxidant Activity using H_2O_2 Free Radical Scavenging Assay

The capacity of the sample extracts to scavenge hydrogen peroxide was monitored according to the method of Ruch *et al*[9]. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4) and 0.6 ml of this solution was added to 3.4 ml of extract aliquots of different concentrations ranging from 10-500 µg/ml. The mixture was then incubated for 10 min and the absorbance of hydrogen peroxide at 230 nm was recorded using a UV spectrophotometer. The percentage of hydrogen peroxide scavenging of *Morus alba* (L.) leaves extracts and standard compounds were calculated:

$$\% \text{ Scavenged } [H_2O_2] = [(AC - AS)/AC] \times 100$$

Where AC is the absorbance of the control and AS is the absorbance in the presence of the sample of *Morus alba* (L.) extracts or standards.

2.6 Antiproliferative Activity of *Morus alba* (L.) Leaves extracts against Breast Cancer Cells

MTT assay was used to determine cell viability based on mitochondrial degradation of tetrazolium dye. The culture medium was prepared with RPMI 1640 media (Himedia Laboratories (Mumbai, India) supplemented with 100 units/ml penicillin and 100 mg/ml streptomycin and 10% heat inactivated FBS. The cells were seeded at a density of 8 x 10^4 cells/well into 96 well plates and allowed to adhere for 24 h in a humidified 5% CO_2 incubator at 37° C. After incubation, cells were treated with different concentrations ranging from 20-350 µg/mL of *Morus alba (L.)* leaves extracts and further incubated for 24 h in a humidified 5% CO_2 incubator at 37° C. Then 10 µl of MTT solution at a concentration of 5mg/ml was added to each well and cells were incubated in a humidified CO_2 incubator at 37 ° C for 3-4 hours in the dark until purple formazan was visible. After incubation, 100 µl of Dimethyl Sulfoxide (DMSO) was added to dissolve purple formazan crystals and kept in the dark for 10-20 minutes. The amount of formazan was determined by measuring the absorbance at 570 nm using an ELISA plate reader (Molecular Devices, Spectramax Plus 384).

The per cent viability was calculated using the formula:

T/C x 100, where T = Absorbance of treatment and C = Absorbance of Control.

2.7 Phytochemical Screening of the Extracts

Screening of the following phytoconstituents: Alkaloids, terpenoids, flavonoids, coumarins and phenols in the ethanolic leaf extract of *Morus alba* (L.) was done using Liquid Chromatography Mass Spectroscopy (LCMS). The LCMS technology involves the use of an HPLC, wherein the individual components in a mixture are first separated followed by ionization and separation of the ions based on their mass/charge ratio. Agilent Advance Bio 6545XT LC/QTOF (Agilent 1290 infinity II) system was equipped with an autosampler, and the employed column was a ZorbaxEclipse Plus C18 1.8 Micrometer, 3 X 100 mm. The mobile phases were water 0.1% formic acid (A) and acetonitrile 0.1% formic acid (B) at a flow rate of 300 µl min^{-1}. The injection volume was 1 µl and the run time was 15 min.

An Attenuated Total Reflection (ATR) technique of Fourier Transform Infrared (FTIR) was used to determine the functional groups in plant extracts by obtaining an infrared spectrum. The FTIR spectra reveal the presence of active constituents ranging from 4000-450 cm^{-1}. The ethanolic leaf extract was subjected to FTIR-ATR to determine the presence of functional groups. The plant extract was prepared in 1:10 ratios and dropped using a capillary on the sensor to characterize the functional groups present in the 4000-450 cm^{-1} range.

2.8 Data Analysis

Data were presented as means ± S.E (Standard Error of the mean) of three independent experiments. The experimental data were analysed using Graph Pad Prism. Differences between groups were tested by one-way ANOVA and F values were calculated at $P \leq 0.05$[10] followed by student 't' test[11].

3. Results

3.1 Estimation of Total Phenolics

Phenolic constituents have a significant role in plants because of their scavenging ability due to their hydroxyl groups[12]. Total phenolics in the ethanolic leaf extract were computed to be 205.30 ± 10.86 µg/mL Gallic Acid Equivalents (GAE) while it was 197.01 ± 5.36 µg/mL and 192.71 ± 10.65 µg/mL GAE in the methanolic leaf extract and aqueous leaf extract respectively as shown in Table 1. These values indicate a higher level of phenolics in the ethanolic leaf extract as compared to the methanolic and aqueous leaf extracts. These results indicate the potential effect of the roles played by phenolic compounds in the

Table 1. Total phenolic content in *Morus alba* (L.) leaves extracts

S. No.	Extract	Total phenolics (mg gallic acid equivalent/g)
1.	MA (Ethanolic)	205.30 ± 10.86*
2.	MA (Methanolic)	197.01 ± 5.36*
3.	MA (Aqueous)	192.71 ± 10.65*

Data represented as means ± S.E of the three replicated experiments and were significant (P < 0.05). Abbreviations: MA = *Morus alba* (L.)

activity of this plant with an expectation of a higher effect in the ethanolic leaf extract.

3.2 Inhibition of H_2O_2 Free Radicals by *Morus alba* (L.) Leaves Extracts

According to the data obtained in Table 2, significant inhibition of H_2O_2 radicals by different concentrations of *Morus alba* (L.) extracts was observed. We have observed a direct positive relationship between antioxidant activity and increasing concentration of the extracts. The relationship was more pronounced in the ethanolic extract than in the methanolic and aqueous extracts. Our results revealed that ethanolic, methanolic and aqueous each extract at 500 µg/ml concentration exhibited 73.35%, 57.35% and 58.10% inhibition respectively, while at the same concentration, ascorbic acid showed 87.3% which was taken as standard. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, free radicals' scavengers, singlet oxygen quenchers, and metal chelators[13]. A higher antioxidant power was shown by the ethanolic leaf extract as compared to the methanolic and aqueous extracts as represented by the calculated IC_{50} values. The recorded IC_{50} values were 293.8 µg/mL, 420.06 µg/mL and 343.35 µg/mL for ethanolic, methanolic and aqueous extracts respectively, whereas it was 248.79 µg/mL for ascorbic acid. As a result, ethanolic leaf extract possessed higher antioxidative potential as compared to other extracts.

Values are the mean ± S.E (n = 3) of three replicated experiments at = Significant at 5% for ANOVA; *P< 0.05 compared with control. Abbreviations: MA = *Morus alba* (L.)

3.3 Effect of *Morus alba* (L.) Leaves Extracts on Proliferation of MCF-7 and MDA-MB-231

Based on the results of the MTT assay, ethanolic leaf extract showed the potential inhibition of cancer cells and reduced the risk of further proliferation. MCF-7 and MDA-MB-231 cell lines were treated with different concentrations of plant leaves extracts ranging from 20 to 350 µg/mL. The viability of the cell lines was recorded after the optimum incubation period (24 h at 37 ˚C). The highest inhibition of cancer cell growth with minimal viability was observed in the ethanolic leaf extract at a concentration of 350 µg/mL, followed by the methanolic and aqueous extracts at the same concentration range. Further, the inhibitory concentration (IC_{50}) of *Morus alba* (L.) extracts on MCF-7 were recorded as 224.6 µg/mL, 241.8 µg/mL and 266.4 µg/mL for ethanolic, aqueous and methanolic extracts respectively as shown in Figure 1. Similarly, the IC_{50} on MDA MB 231 was calculated as 183 µg/mL, 212.6 µg/mL and 213.5 µg/mL for ethanolic, methanolic and aqueous respectively after 24 h at 37˚C as shown in Figure 2. Our results agree with previous reports showing the DCM (Dichloromethane) extract of *Morus alba* (L.) possessed higher antiproliferative activities against human cancer cells HCT-116 and MCF-7[14]. Thus, the ethanolic leaf extract was found to have higher anticancer potential than methanolic and aqueous leaf extracts against breast cancer cells.

Table 2. Hydrogen peroxide scavenging activity of Vitamin C and *Morus alba* (L.) leaves extracts

Concentration (µg/ml)	Vitamin C	Ethanolic (MA)	Methanolic (MA)	Aqueous (MA)
10	17.01 ± 0.03*	10.41 ± 0.08	18.07 ± 0.05*	11.42 ± 0.01*
20	22.86 ± 0.01*	17.39 ± 0.02*	23.23 ± 0.03*	26.61 ± 0.04*
50	36.33 ± 0.03*	29.13 ± 0.03*	27.16 ± 0.03*	36.09 ± 0.01*
100	40.79 ± 0.05*	42.11 ± 0.02*	34.79 ± 0.03*	40.98 ± 0.03*
200	52.44 ± 0.01*	54.15 ± 0.02*	41.86 ± 0.03*	49.03 ± 0.02*
400	68.90 ± 0.01*	61.41 ± 0.02*	45.34 ± 0.04*	56.07 ± 0.03*
500	87.30 ± 0.02*	73.35 ± 0.01*	57.35 ± 0.04*	58.10 ± 0.03*
F values at 5% Level	83.06@	106.23@	249.34@	451.53@

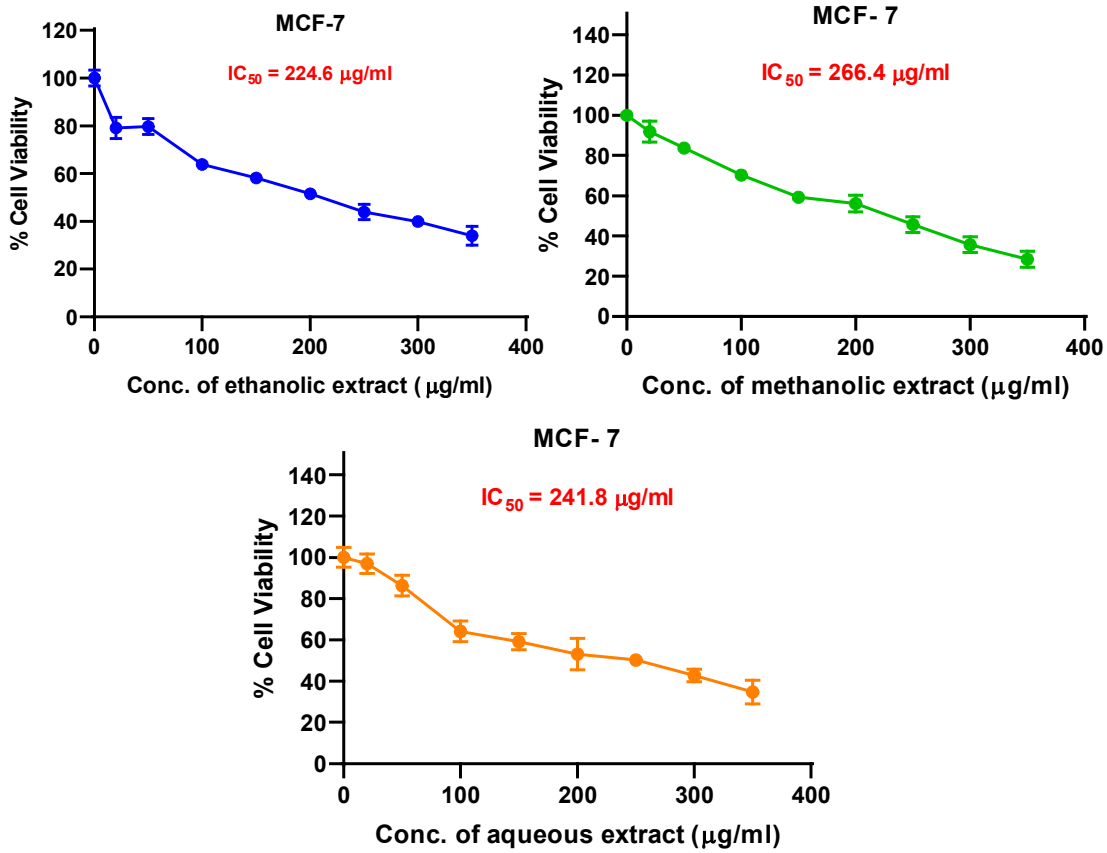


Figure 1. The antiproliferative effect of different extracts of *Morus alba* (L.) leaves on human breast cancer cells (MCF-7). Data represented as means ± S.E three replicated experiments and were significant ($P < 0.05$).

3.4 Phytochemical Analysis of Ethanolic Leaf Extract of *Morus alba* (L.)

The LC-MS chromatogram of the ethanolic leaf extract of *Morus alba* (L.) is shown in Figure 3. It was observed that the different peaks were obtained at different retention times. The important compounds identified in the ethanolic leaf extract of *Morus alba* (L.) are given in Table 3.

3.5 Fingerprinting of Functional Groups using FT-IR

The presence of various functional groups in the ethanolic leaf extract was determined by FTIR. It records the molecular structure and chemical bonds of the compound in graphical form for the identification of the class of compound in the extracts. The results of FTIR analysis of the extract confirmed the presence of O-H, C=C, C-H, C-O and iodo functional groups as shown in Figure 4 and listed in Table 4.

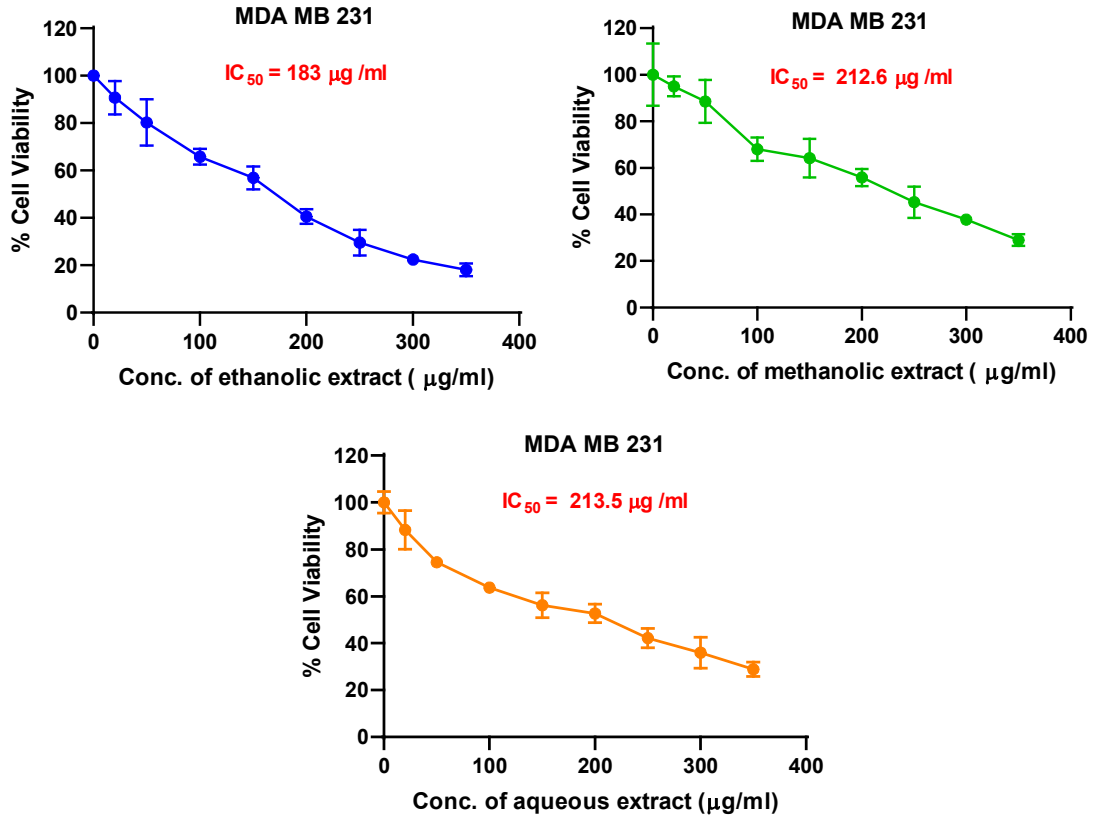


Figure 2. The antiproliferative effect of different extracts of *Morus alba* (L.) leaves on human breast cancer cells (MDA-MB-231). Data represented as means ± S.E of the three replicated experiments and were significant ($P < 0.05$).

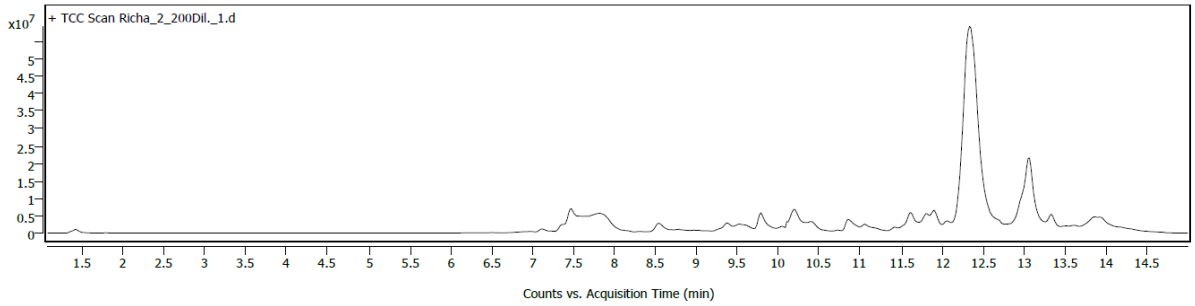


Figure 3. LC-MS chromatogram of *Morus alba* (L.) ethanolic leaf extract.

Table 3. Important compounds identified from the ethanolic leaf extract of *Morus alba* (L.) by LC-MS

S/No.	Name	Formula	Mass	RT	Score
1.	Glucopyranosylmoranoli Ne	C12 H23 N O9	325.1375	1.346	68.08
2.	1-Deoxynojirimycin	C6 H13 N O4	163.0845	1.352	87.61
3.	Kaempferol 3 -O-? Dgalactoside	C21 H20 O11	448.1006	4.715	67.72
4.	Linalool	C10 H18 O	154.1358	8.592	91.14
5.	Isocyclocitral	C10 H16 O	152.1203	9.893	87.28
6.	trans Resveratrol	C14 H12 O3	228.0791	10.424	79.90
7.	m-Coumaric acid	C9 H8 O3	164.0472	11.143	81.97
8.	3,4- Methylenedioxybenzoic Acid	C8 H6 O4	166.0266	12.326	98.95

Table 4. Fingerprinting of functional groups of *Morus alba* (L.) ethanolic leaf extract

S. No.	Wave Number (cm-1)	% Transmittance	Assignments	Possible Molecule Type
1	3293	78.2	Stretching O-H symmetric	Hydroxy group, H-bonded OH stretch
2	2926	70.8	Methylene C-H asymmetry STRETCH	CH_2
3	1800	100.18	Aryl carbonate	CO_3^{-2}
4	1367	74.09	Phenol or secondary, OH bend	C_6H_5OH
5	1285	77.68	Phenol or secondary, OH in-plane bend	C_6H_5OH, OH
6	1041	45.57	Primary alcohol, C-O stretch	C_2H_5OH
7	988	49.91	Vinyl C-H out-of-plane bend	C-H
8	923	70.39	Vinyl C-H out-of-plane bend	C-H
9	817	76.56	C-H 1,4-Disubstitution (para)	C-H
10	520	53.12	Aliphatic iodo compounds	Iodo compounds

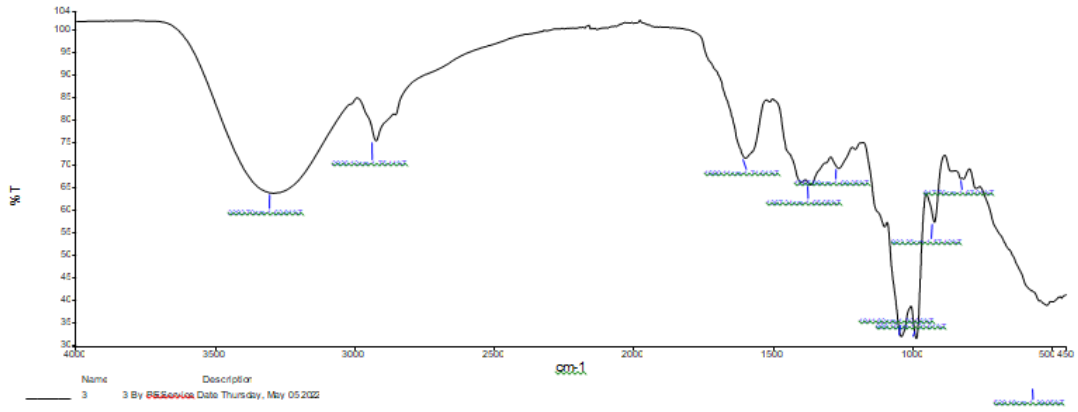


Figure 4. FTIR spectrum of *Morus alba* (L.) ethanolic leaf extract showed the peaks of various functional groups. The spectrum was recorded in the range of 4000-450 cm^{-1}.

4. Discussion

Mulberries are a good nutritional source of a variety of phenolic compounds, like flavonols and phenolic acids in black and red mulberry[15]. Previous studies have elaborated the antiproliferative effect of methanol extracts from mulberry plant parts on human cancer cell lines (Calu-6 for human pulmonary carcinoma; SMU-601 for human gastric carcinoma, MCF-7 for human breast adenocarcinoma and HCT-116 for human colon carcinoma) and found significantly effective in inhibiting the cell growth[16]. The antiproliferative action of *Morus alba* (L.) methanolic leaf extract against breast cancer cells causing apoptosis by downregulation of iNOS has been reported[17]. Here, the present findings demonstrated similar results where *Morus alba* (L.) leaves extract significantly inhibited the proliferation of breast cancer cells (MCF-7 and MDA MB 231) in a dose-dependent manner. The extracts were shown to inhibit the cell growth in a concentration-dependent manner and the lower IC_{50} value corresponds to the maximum cytotoxicity potential of treatment against a particular cell line. We have found that ethanolic leaf extract of *Morus alba* (L.) was found to be highly cytotoxic against MCF-7 and MDA-MB-231 at the concentration of 350 µg/mL with 66% and 82% growth inhibition respectively. Thus, we can conclude that the ethanolic leaf extract of *Morus alba* (L.) showed the highest antiproliferative activity against breast cancer cells.

The species Morus is a rich source of polyphenols, flavonoids, terpenes, and alkaloid-type metabolites such as 1-deoxynojirimycin[18] which have been shown to possess antioxidant properties. These phytochemicals are capable of scavenging free radicals produced in the body during stress conditions. It has been reported that the H_2O_2 scavenging abilities of the extracts may be due to the structural features of their active components, which determine their electron-donating capabilities[19].

The antioxidant activity of different extracts of *Morus alba* (L.) was determined using an H_2O_2 free radical scavenging assay and scavenging of H_2O_2 by the extracts may be attributed to their phenolics, which can donate electrons to H_2O_2, thus neutralizing it to water[20]. Our results show a decrease in the concentration of OH* radicals due to the scavenging ability of the soluble constituents in the ethanolic, methanolic and aqueous extracts of *Morus alba* (L.) Our results showed the antioxidative activity of *Morus alba* (L.) against H_2O_2 in a concentration-dependent manner. The maximum percentage of inhibition of H_2O_2 radicals was found in the ethanolic leaf extract followed by aqueous and methanolic leaf extract.

The total phenolic content of the tested plant extracts was highly correlated with the radical scavenging activity. The total phenolic content was determined and found to be highest in the ethanolic leaf extract followed by methanolic and aqueous leaf extracts. We further investigated the phytoconstituents present in the ethanolic leaf extract which might be responsible for enhancing its potency, as shown in biochemical assays. Concerning the above FTIR spectrum of ethanolic leaf extract of *Morus alba (*L.) in Figure 4. The peaks between 4000 to 450 cm^{-1} correspond to different chemical bonds and functional groups and these different peaks confirm the presence of hydroxyl group, alkenes, and aliphatic compounds. Moreover, LC-MS helps in identifying the main compounds using their molecular weight in the ethanol leaf extract as listed in Table 3. The identified compound Kaempferol 3-O-β-D galactoside, commonly known as astragalin is a flavonoid exhibiting anti-inflammatory, antioxidant, and anti-atopic dermatitis potential[21]. The other identified compound was trans- Resveratrol, which is a polyphenol having anticarcinogenic, antiviral, neuroprotective, anti-inflammatory and antioxidant properties[22]. Another important bioactive constituent was m-Coumaric acid which is a phenolic acid that possesses antioxidant and antimicrobial properties. The extract also shows the presence of terpenoids (Linalool, Isocyclocitral), iminosugar (1-Deoxynojirimycin) and organic compound (3,4-Methylenedioxybenzoic acid) in the extract exhibiting various biochemical properties.

5. Conclusion

Based on the results, all the extracts have significant antioxidant activity compared to ascorbic acid taken as standard. Moreover, the ethanolic leaf extract showed higher antioxidant and antiproliferative activities followed by methanolic and aqueous extracts and thus can be useful as a food supplement and in the pharmaceutical industry. In addition, the antioxidant and antiproliferative activity may be due to bioactive compounds identified in the extracts. Therefore, it is suggested that further work be performed on the isolation and characterization of these bioactive constituents to explore their biochemical

properties. Thus, it can be concluded that *Morus alba* (L.) leaves possess anticancer activity against breast cancer cells and may be exploited as a promising therapeutic option in developing anticancer drugs.

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