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RESEARCH ARTICLE

Antioxidant and antiproliferative properties of *Moringa oleifera* Lam. leaf aqueous extract

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ABSTRACT
Moringa oleifera Lam. is a highly valued medicinal plant in India, especially Kerala. In the present study, antioxidant activity of aqueous extract of leaves of *M. oleifera* was determined both *in-vitro* and *in-vivo*. Male Wistar rats of 3 age groups- 6, 12, and 18 months old were used for *in-vivo* analysis. *In vitro* anti-proliferative effect of the extract was carried out in Dalton's Lymphoma Ascites (DLA) Cells. LCMS-QTOF analysis of the extract was also done to determine the bioactive components present in the extract. Antioxidant activity of *M. oleifera* leaf showed an IC 50 value of 10.47 µg/ml and whereas for standard drug, ascorbic acid, it was 19.52 µg/ml. *In-vivo* analysis of lipid peroxidation showed a significant reduction of lipid peroxidation in the brains of 12 and 18-months old treated groups. Up to 75% mortality of DLA cancerous cells was observed *in-vitro* in different concentrations of *M. oleifera* leaf water extract in a dose-dependent manner, demonstrating its anti-proliferative property. LCMS-QTOF analysis revealed the presence of emodin-8-glucoside in the extract. Molecular docking analysis (Auto Dock Vina) of emodin-8-glucoside with six cancer related proteins showed highest binding affinity with AKT-1 with a binding score of -10.4 kcal/mol, also showed good affinity with NF-kB (p65), Stat-3, Bcl-2, Bcl-xl and c-FLIP. This study helps to choose healthy diet practices to overcome free radical onslaught and cancerous cell proliferation especially in the later stages of life. This can also pave way for the emergence of diet based therapeutic cure for cancer.

Introduction

Studies of pharmacological applications of medicinal plants used in the diet entails a new arena of research. Several plants are being investigated for their pharmacological potential. There is need for research to understand the nontoxicity and economic viability of these medicinal plants being used for various pharmacological interventions, especially to fight against cancer (1).

Moringa oleifera Lam. (Family Moringaceae), is also called as horseradish tree, drumstick tree in English and in Sanskrit, it is called as Shigru. It has been an ingredient of our diet for several centuries. Its reported effects include: antitumor (2, 3), hypotensive (4), antifungal (5), antispasmodic and anti-inflammatory activities (6). Bioactive compounds present in *M. oleifera* include vitamins, carotenoids, polyphenols, flavonoids, essential amino acids and phenolic acids. All the parts of the tree are edible and possesses various pharmacological actions and therefore, used in the Indian traditional medicine to cure to various ailments; and is called 'miracle tree' (7-9).

In the present study, *M. oleifera* leaf aqueous extract was investigated for its antioxidant activity and anti-proliferative activity. LCMS QTOF analysis was also conducted to determine the bioactive components present in the extract and *in silico* molecular docking analysis was also performed using emodin-8-glucoside with six anti-cancer targets. Emodin-8-glucoside is a potential candidate for anti-cancerous activity as it is an anthraquinone and has got a striking chemical resemblance to anthracyclines as clinically well-established anticancer drug (10-12).

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Materials and Methods

Plant material collection and authentication

M. oleifera leaves were collected during the months of April-May 2018 from homestead garden (10°01'53.5" N 76°29'15.2" E), Ernakulam, Kerala, India. and was shade dried for 7 days and powdered, sieved and stored in sterile glass bottles in refrigerator until use. The plant material was authenticated (authentication no. NISCAIR/RHMD/Consult/ 2019/3403-04) by CSIR-NISCAIR RHMD (Raw Material Herbarium and Museum, New Delhi). The identification was done on the basis of macroscopic studies of the sample followed by scrutiny of literature and matching the sample with authentic samples deposited in the RHMD. DNA barcoding (*matk* gene; GenBank sequence ID: MK165485.1) of the sample was done at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala, India. The voucher specimen (No. 486) and Herbarium was deposited at Dept. of Botany, St. Albert's College Herbarium (SAC) Ernakulam, Kerala, India.

Plant extract preparation

Soxhlet extraction was carried out by taking 50 gm of dried *M. oleifera* leaf powder in 500 ml distilled water. The extract was concentrated using rotary evaporator (Hahnvapor HS2005V, Hahnshin Scientific) and lyophilised under vacuum. The extract thus obtained was used for further studies.

Percent yield

The percent yield was calculated after running the Soxhlet extraction process for a fixed period of time (48 hrs). It was calculated by taking the weight of the extract obtained after lyophilization with the initial weight of the leaf powder taken for the extraction process, using the equation:

$$\% \; yield = \frac{\text{Weight of dry extract} \times 100}{\text{Weight of powdered leaves}}$$

Preliminary phytochemical screening

Qualitative analysis of *Moringa oleifera* leaf extract was done for qualitative identification of various phytochemical constituents such as carbohydrates, tannins, saponins, flavonoids, quinones, glycosides, cardiac glycosides, terpenoids, phenols, coumarins, steroids, phytosteroids and anthraquinones with standard methods (13-15).

Antioxidant activity in-vitro

Antioxidant activity of the lyophilized aqueous extract of *M. oleifera* leaf in various concentrations ranging from 20 µg/ml to 200µg/ml was tested by DPPH method (16). Ascorbic acid was used as the standard. Statistical analysis (one-way ANOVA) and IC50 values were calculated using GraphPad Prism (Ver 5.0).

In-vivo lipid peroxidation study

Healthy male Wistar rats belonging to three age groups; 6 months old, 12 months old and 18 months old were selected for the study. After the acclimatization period of two weeks, the animals were divided randomly in to control and treatment groups of 6 animals each. IAEC approval for the study was obtained from the College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India (Order No. Acad(3)/6554/04, dated 27/09/18). Lyophilized aqueous extract of *M. oleifera* leaf was given orally at a dosage of 200 mg/kg body weight (17, 18) for 30 days to all the treatment group animals. The corresponding control animals received same volume of distilled water orally as that of the treatment group. During the study, the animals were housed in standard-sized polypropylene cages and were fed with water and commercial pelleted feed *ad-libitum*. On the 31st day, all the animals were sacrificed by CO_2 euthanasia and brain was removed immediately and weighed. The brain sample was homogenized in normal saline and centrifuged at 3000 rpm. The supernatant thus obtained was used for lipid peroxidation assay. Lipid peroxidation was determined by the standard method (19). To 1 ml of the sample, 2 ml 1:1:1 TBA – TCA – HCl (Acetic acid – 15%; Thiobarbituric acid in hot distilled water – 0.38% and HCl – 0.25N) reagent was added and mixed well and heated in a boiling water bath for 15 min. After cooling, the mixture was centrifuged at 3200 rpm for 15 min. The supernatant was taken and absorbance was read at 535 nm in Perkin Elmer UV/VIS/NIR spectrometer Lambda 750. Lipid peroxidation was expressed as nmoles of MDA/mg tissue. Tetra-methoxy propane (4.8 Mm) was used as standard.

In-vitro anti-proliferative study

The lyophilized aqueous extract of *M. oleifera* leaf was tested for its anti-proliferative property in Dalton's Lymphoma Ascites cells (DLA) (20). The tumour cells aspirated from the peritoneal cavity of tumour bearing mice were washed thrice with Phosphate Buffer Saline (PBS). Cell viability was determined by trypan blue exclusion method. Viable cell suspension (1×10^6 cells in 0.1 ml) was added to tubes containing various concentrations (10 µg/ml to 200 µg/ml) of lyophilized aqueous extract of *M. oleifera* leaf and the volume was made up to 1 ml using PBS. Control tubes contained only cell suspension. These assay mixtures were then incubated for 3 hrs at 37 °C. Cell viability after the drug treatment was assessed by mixing with 0.1 ml 1% of trypan blue and keeping for 2-3 min. Then it was loaded on the counting chamber of haemocytometer and the number of dead and live cells were determined. The number of stained and unstained cells were counted separately and percentage cytotoxicity was determined. The study was conducted in triplicate and the statistical analysis (One Way ANOVA) was carried out using GraphPad Prism (Ver 5.0).

LCMS QTOF analysis (liquid chromatography mass spectrometry - quadrupole time-of-flight)

LCMS QTOF analysis was done over an LCMS instrument (Agilent Technologies, USA) after the digestion and desalting of protein part from the lyophilized aqueous extract of *M. oleifera*. Details of the instrumentation are as follows: Hypersil GOLD C18 100 x 2.1 mm^{-3} MICRON, Hip Sampler, Binary

pump and Q-TOF. For MS Q-TOF (G6550A), the ion source used was Dual AJS ESI. The eluent was monitored by electrospray ion mass spectrometer (ESI-MS) under positive-ion mode. ESI was conducted by using a fragmentor voltage of 125 V, skimmer 65 V, gas temperature at 250 °C and nebulizer at 35 psi. Running time was 30 min. The mode of sample application to Hip Sampler was by injection with needle wash and the injection volume was 10.00 µL. The high-pressure limit in the binary pump (G4220B) was 1200 bar and the flow rate were 0.3 ml/min. Data analysis was processed with Agilent Mass Hunter and acquisition method used was 30 mins_+ESI_10032014_MS-MS.m. The composition of solvents is given in (Table 1). The analysis was done

Table 1. Solvent composition of LCMS-QTOF Analysis

	Solvent name	Channel 2 solvent	%
A	0.1% FA in water	100.0 % Water V.02	95%
B	90% ACN +10% H2O+ 0.1% FA	100.0 % Acetonitrile V.02	5%

at Sophisticated Analytical Instrumentation Facility (SAIF) at IIT Bombay, India.

In-silico analysis

Molecular docking analysis was done to determine the anti-proliferative potential of emodin-8-glucoside against six cancer signalling proteins such as AKT1 (PDB ID:3O96), Bcl-2 (PDB ID: 5JSN), NF-kB (p65) (PDB ID: IK3Z), Stat-3 (PDB ID: 6NUQ), Bcl-xl (PDB ID: 2YXJ) and c-FLIP (PDB ID: 3H13). PDB structure of the proteins were downloaded from RCSB-PDB (https://www.rcsb.org/) and the structure of the ligand emodin-8-glucoside (PubChem ID: 99649) was downloaded from PubChem database. Protein preparation were done using PyMOL (Schrodinger) software, where heteroatoms were removed and hydrogen atoms were added to all the proteins. Docking analysis was done using AutoDock Vina (21) and the docked poses were visualized using Discovery Studio visualizer. Grid box parameters for AKT-1 was X = 6.299, Y = -7.951 and Z = 17.275, for Bcl-2; X = -3.509, Y = -5.511 and Z = 58.241, for NF-kB; X = 15.566, Y= -0.545 and Z = -0.955, for stat-3; X = -2.217, Y = 19.174 and Z = 24.597, for Bcl-xl; X = -7.138, Y = -21.012 and Z = 1.74 and for cFlip; X = 35.338, Y = -34.637 and Z = -14.5.

Statistical analysis

The statistical significance was determined by one-way ANOVA with Tukey's multiple comparison test using GraphPad Prism Ver. 5. P value <0.05 was considered significant.

Results

Percent yield and preliminary phytochemical screening

Percent yield for lyophilized aqueous extract of *M. oleifera* leaf was 30% (Table 2). Preliminary phytochemical screening of the extract showed the presence of flavonoids, carbohydrates, coumarins, saponins and terpenoids (Table 3).

Table 2. percent yield of aqueous extract of *M. oleifera* leaf

Name of the extract	Percent yield (%)
M. oleifera leaf aqueous extract	30±2 %

Table 3. Preliminary phytochemical screening of aqueous extract of *M. oleifera* leaf

Sl. No.	Phytochemicals	Test name	Observation	*M. oleifera* leaf extract
1	Carbohydrates	Benedict's Test	Orange red precipitate	+
2	Flavonoids	Alkaline Reagent Test	Yellow colour	++
3	Quinones	With Conc. H_2SO_4	Red colour	-
4	Glycosides	Borntrager's test:	Pink colour	-
5	Cardiac glycosides	Keller- Killiani's Test	Brown ring	–
6	Terpenoids	Salkowski test	Brown colour	+
7	Phenols	Ferric Chloride Test	Blue or green colour	+
8	Coumarins	With 10% NaOH	Yellow colour	++
9	Phlobatannins	With 2% HCl	Red colour precipitate	–
10	Anthraquinones	Borntragers test	Pink colour precipitate	–
11	Tannins	Ferric chloride Test	Dark blue or green	–
12	Saponins	Frothing test	Fam formation	+
13	Steroids & Phytosteroids	Libermann Burchard's test	Brown Ring	+

'+' indicates the presence and '- 'indicates the absence

Antioxidant activity in-vitro

The lyophilized aqueous extract of *M. oleifera* leaf showed higher antioxidant activity than the ascorbic acid at both the concentration of 10 µg/ml (48.20± 0.4%) and 20 µg/ml (82±0.5%), while at the concentration of 50 µg/ml, the extract showed 93.20±0.3% whereas the ascorbic acid showed 94.54±0.6% inhibition of DPPH radical. The IC_{50} value for the *M. oleifera* leaf extract was 10.47 µg/ml while it was 19.52 µg/ml for ascorbic acid (Table 4).

Table 4. Percentage inhibition of DPPH radical by *M. oleifera* leaf lyophilized aqueous extract and ascorbic acid

Concentration (µg/ml)	% Inhibition (Mean±SD)	
	M. oleifera leaf extract	Ascorbic Acid
10	48.20±0.40%	35.78±0.35%
20	82.00±0.50%	67.78±0.43%
30	90.00±0.70%	92.87±0.56%
40	91.50±0.72%	94.08±0.68%
50	93.20±0.60%	94.54±0.72%
IC_{50}	10.47 µg/ml	19.52µg/ml

Lipid peroxidation in-vivo

The treatment with *M. oleifera* leaf water extract significantly ($p<0.05$) reduced lipid peroxidation in the brains of 12- and 18-months old rats after 30 days treatment. But the 6-months old treated group did not

Table 5. Levels of TBARS in the brain tissue of Wistar rats treated with *M. oleifera* leaf water extract at a dosage of 200 mg/kg body wt. (Mean ± SEM) (n=6)

Age (months)	Lipid Peroxidation (nM / mg tissue)	
	Control group	Treatment group
6	3196.67±206[a]	3001±159[a]
12	4442.66±112[b]	2431.66±118[a]
18	5388±50[c]	2641±200[a]

One-way ANOVA with repeated measures Tukey's multiple comparison test using Graph Pad Prism (Ver 5.0). Groups with same superscript did not vary significantly (P<0.05).

show any significant change when compared to the control group (Table 5).

In-vitro anti-proliferative activity

Various concentrations of the lyophilized aqueous of *M. oleifera* ranging from 10 µg/ml to 200 µg/ml extract were studied and a substantial suppression of 75 ±0.4% and 65±0.5 % of DLA cancerous cells was obtained at a concentration of 200 µg/ml and 100 µg/ml, respectively. A concomitant decrease in the activity was also observed as the concentration decreased. According to Tukey's honest significance

Table 6. Anti-proliferative activity of *M. oleifera* leaf water extract.

Sl. No.	Concentration of *M. oleifera* leaf water extract (µg/ml)	Anti-proliferative activity (% death of cancerous cells) mean±SD
1	200	75[a]±3.46
2	100	65[b]±3.28
3	50	50[c]±3.03
4	20	40[d]±3.03
5	10	35[e]±2.9

One-way ANOVA with repeated measures Tukey's multiple comparison test using Graph Pad Prism (Ver 5.0). Groups with same superscript did not vary significantly (P<0.05).

test, all the treatment groups except between 20 µg/ml and 10 µg/ml groups were statistically highly significant (P<0.05) (Table 6).

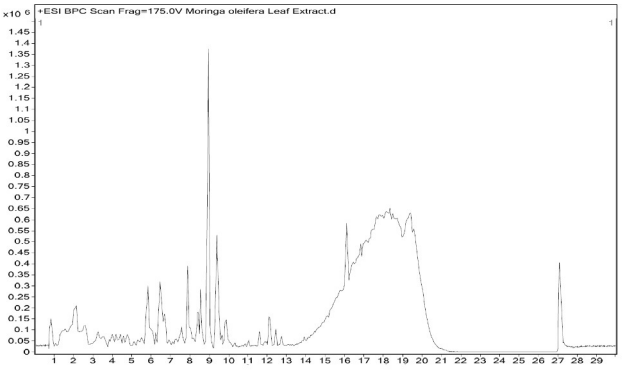

Fig. 1. Chromatogram of LC/MS Analysis of *M. oleifera* leaf water extract.

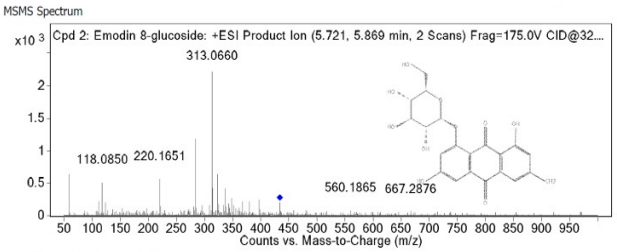

Fig. 2. MSMS Spectrum of Emodin-8-glucoside.

LCMS -QTOF analysis

LCMS analysis (Table 7, Fig. 1 & 2) revealed the presence of emodin 8 glucoside along with 11-amino-undecanoic acid, some glycerophosphoethanolamines (GPEtn) and fatty acids with glycerol were also identified.

Molecular docking analysis

Molecular docking studies revealed the binding affinity of emodin-8-glucoside (Fig. 3a-f) with AKT-1, NF-kB (p65), Stat-3, Bcl-2, Bcl-xl and c-FLIP. With AKT-1 emodin-8-glucoside had the most negative binding affinity. The binding affinity was -10.5 kcal/mol for AKT-1and forBcl-xl and stat3, it showed -8.3 kcal/mol and -7.6 kcal/mol, respectively. Lowest binding affinity was for Bcl-2 with a binding score of -7.2 kcal/

Table 7. Result of LCMS QTOF analysis of *M. oleifera* leaf aqueous extract

No.	Compound Label	RT	Mass	Formula	Hits
1	Emodin 8-glucoside	5.795	432.1	C21 H20 O102	8
2	His Ser His	8.069	379.15	C15 H21 N7 O5	10
4	11-amino-undecanoicAcid	9.517	201.173	C11 H23 N O2	3
6	GPEtn(20:0/17:0)[U]	16.21	761.58	C42 H84 N O8 P	9
7	GPEtn(22:0/26:0)[U]	17.09	915.773	C53 H106 N O8 P	5
8	1-hexadecanoyl-2-(9Zhexadecenoyl)-3octadecanoylsn-glycerol	18.46	832.74	C53 H100 O6	10
9	TG(16:1(9Z)/20:0/20:3(8Z,11Z,14Z))[iso6]	18.49	910.7	C59 H106 O6	10
10	1-heptadecanoyl-(9Z,12Zheptadecadienoyl)-3-octadecanoyl-sn-glycerol	18.51	858.76	C55 H102 O6	10
11	1-hexadecanoyl-2-(11Z-octadecenoyl)-sn-glycerol	19.04	594.51	C37 H70 O5	10
12	TG(17:1(9Z)/17:2(9Z,12Z)/22:3(10Z,13Z,16Z))[iso6]	19.86	905.76	C59 H102 O6	10

Table 8. Showing the binding energy and interactions of Emodin-8 glucoside with various cancer targets

Targets	Binding Energy	Interactions
AKT-1	-10.5	Van der Waal's interactions: Gln-79, Tyr-272 and Ile-290, conventional hydrogen bond: Thr-211, carbon-hydrogen bond: Ser-205, pi-anion interaction: Asp-292, Pi-Pi stacked interaction: Trp-80, alkyl and Pi-alkyl interactions: Leu-264, Lys-268 and Val-270 and Leu-210
NF-*k*B	-7.4	Van der Waal's interaction: Ser-261, Ala-264, Thr-292, Asp-293, Arg-297 and Glu-299, conventional hydrogen bonds: Gln-263, Asp-291 and His-296, alkyl and pi-alkyl interactions: Pro-265 and Pro-290
Stat-3	-7.6	Van der Waal's interaction: Gln-247, Ala-250, Cys-251, Ile-252, Gly-253, Glu-324, Arg-325, Gln-326, Cys-328, Arg-335, Pro-336, Trp-474, conventional hydrogen bond and Pi-anion interaction: Asp-334, carbon hydrogen bond with Ser-514, alkyl and pi-alkyl interactions: Pro-256. Unfavourable acceptor-acceptor interaction Pro-333
Bcl-2	-7.2	Van der Waal's interaction: Asp-111, Met-115, Glu-136, Arg-139, Asn-143, and Phe-153, conventional hydrogen bond: Asp-140, pi-sigma with Phe-112, Pi-Pi T-shaped interaction: Tyr-108 and Phe-104 and Pi-alkyl interaction: Ala-149
Bcl-xl	-8.3	Van der Waal's interaction: Phe-97, Asp-107, Phe-131, Asp-133 and Phe-146, conventional hydrogen bonds: Leu-130 and Arg-139, carbon-hydrogen bond and Pi-anion interaction: Glu-129, alkyl and pi-alkyl interactions: with Ala-104, Phe-105, Leu-108, Ala-142 and Ala-149

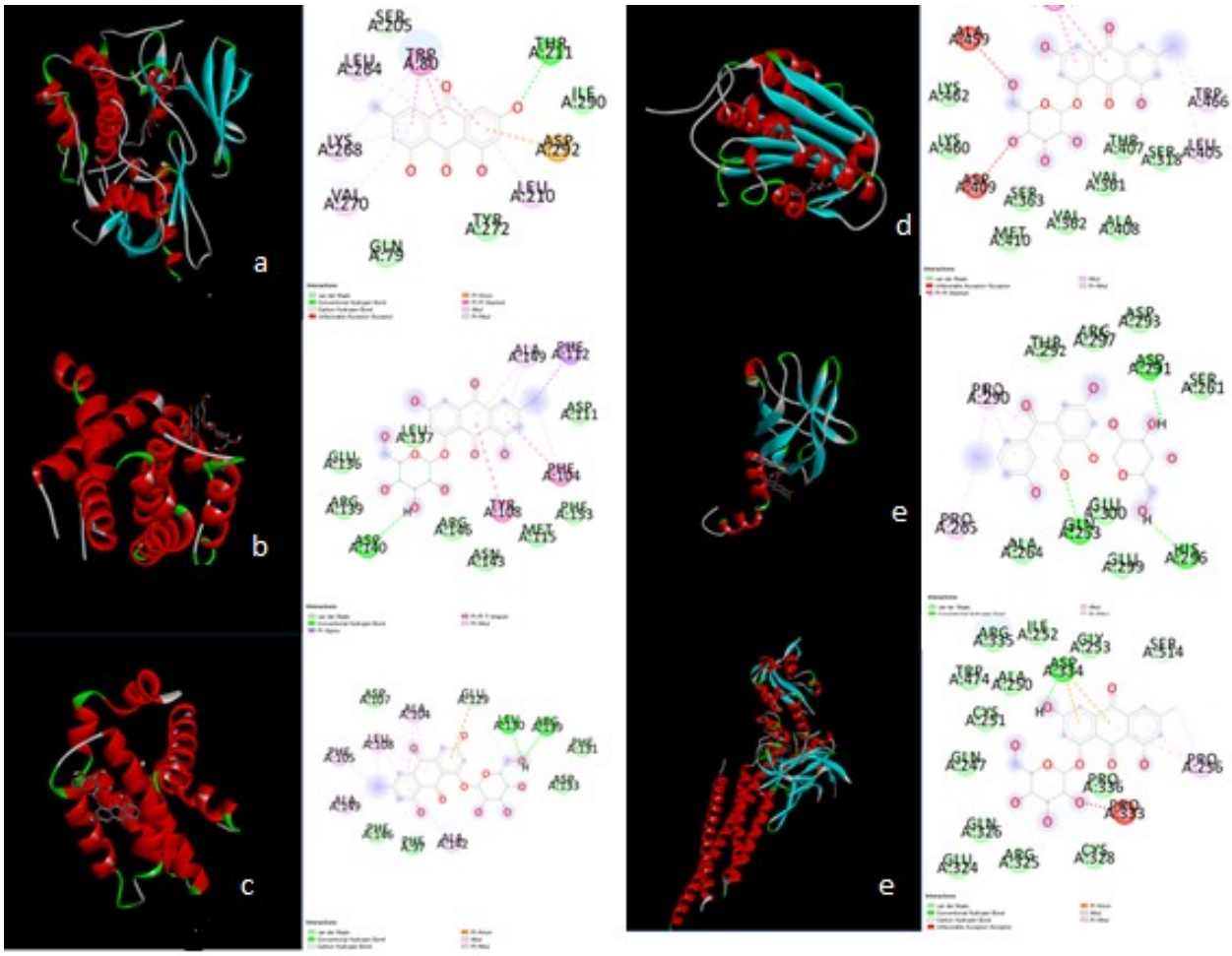

Fig. 3. a-f. 2D Images of binding of emodin-8-glucoside with AKT-1 (**a**), Bcl-2 **(b)**, Bcl-xl **(c)**, c-FLIP **(d)**, NF-kB (p65) **(e)**, Stat-3 **(f)**.

mol. With cFLIP and NF-kB(p65), emodin-8-glucoside showed a binding affinity of -7.3 kcal/mol and -7.4 kcal/mol, respectively (Table 8, Fig. 3a-f).

Discussion

Percent yield and preliminary phytochemical screening

The percent yield of an extract defines the efficiency of the extraction process and here we got 30% yield when water was used as the solvent for the extraction process (Table 2). High yields of water extract accounts for hydrophilic nutrients, carbohydrates amino acids, water soluble secondary metabolites, nutrients, electrolytes and phenolics to some extent etc., which are insoluble or slightly soluble in the presence of ethanol (22). It was also reported a percent yield of 37.83% with water (23). Preliminary phytochemical screening analysis were done to determine various phytoconstituents present in *M. oleifera* leaf aqueous extract. All parts of *M. oleifera* have been used for medicinal purposes and it is imminent that the phytoconstituents present in the plant is responsible for the medicinal value. One study reported the presence of alkaloids, saponins, tannins, flavonoids and sugars from aqueous extracts of *M. oleifera* leaf (24). In the present study also, water extract showed the presence of flavonoids,

carbohydrates, coumarins, phenols, saponins, terpenoids, steroids and phytosteroids (Table 2).

In-vitro antioxidant activity

M. oleifera is reported to contain greater amounts of flavonoids than any other vegetable or fruit (25). In the present study, *M. oleifera* leaf aqueous extract exhibited 93.20% inhibition of DPPH radical at a concentration of 50 µg/ml and the IC_{50} value was found to be 10.47 µg/ml (Table 4). Most of the flavonoids reported in plants are water soluble and are responsible for antioxidant activity in water extracts thus providing protection against oxidative damage (26). In the present study too, greater antioxidant activity comparable to vitamin C was obtained with the *M. oleifera* leaf extract. *M. oleifera* leaves can be considered as dietary supplement for healthy ageing and to prevent age associated free radical onslaught, especially, in the brain. Sreelatha and Padma (26) reported that water extracts of both the tender leaves and mature leaves of *M. oleifera* have significant protective effect against oxidative damage by reducing lipid peroxidation, inhibiting superoxide anion, nitric oxide and complete reversal of H_2O_2 induced DNA damage. Earlier *in-vitro* studies also reported protective effect of extracts of Moringa leaves against oxidative stress (27, 28). Similar results were also reported by Charoensin, 2014 in *M. oleifera* methanol and dichloromethane extracts (22).

Lipid peroxidation in-vivo

The finding that the extract significantly reduced lipid peroxidation in 12 months old and 18 months old experimental group animals shows the efficient antioxidant property of Moringa leaves (Table 5). The lipid peroxidation expressed in malondialdehyde (MDA) levels can be considered as a direct measure of cellular oxidative damage (29), the significant decrease in MDA levels in aged animals was due to the antioxidant role played by the extract. Since lipid peroxidation is one of the major causes of ageing, its prevention offers a better protective measure against many neurodegenerative diseases also. Protection against lipid peroxidation can also maintain cellular integrity and, thus, can restore cellular homeostasis during the process of ageing. Highly significant increase in lipid peroxidation was seen with age of the rats which were used as the control in the present study. It is to be noted that all the treated groups of Wistar rats showed similar lipid peroxidation levels of 6 months old control animal irrespective of the age of the treated animals. The bioactive components having antioxidant property can be responsible for scavenging free radicals and chelation of transition metal ions, which accelerates lipid peroxidation via Fenton reaction (30). Treatment with *M. oleifera* leaf hydroalcoholic extracts has been reported to significantly reduce lipid peroxidation in cortex, hippocampus and striatum (31). Pre-treatment with *M. oleifera* leaf methanol extract at a dosage of 250 and 500 mg/kg significantly attenuated chlorpyrifos induced lipid peroxidation in rat brain and also reduced neuronal degeneration (32). The bioactive component emodin-8-glucoside identified via LCMS QTOF (*vide infra*) could be one potent free radical scavenger in *M. oleifera* extract and as responsible for the reduction of lipid peroxidation in the present study. The present study, thus establishes the antioxidant activity of *M. oleifera* through *in-vivo* studies too.

In-vitro anti-proliferative activity

Even though all parts of the *M. oleifera* plant have been extensively studied for their respective anti-cancer property, only few compounds have been identified from water extract, compared to methanolic or ethanolic extracts. Tiloke and coworkers (33) have done antiproliferative effect of *M. oleifera* crude aqueous leaf extract on human esophageal cancer cells (SNO) and observed that the extract significantly increased lipid peroxidation and DNA fragmentation in SNO cells. They found that the induction of apoptosis was mediated by the increase in phosphatidylserine externalization, caspase-9 and caspase-3/7 activities and decreased ATP levels. In addition, the extract significantly increased both the expression of Smac/DIABLO protein and cleavage of poly [ADP-ribose] polymerase 1 (PARP-1), resulting in an increase in the 24 kDa fragment (33). According to one study, *M. oleifera* leaf extract was more potent than callus extract in reducing the viability of HeLa cervix cancer cells in a concentration-dependent manner (34). Our study is in line with these results that we also got a concomitant increase in cancerous cell death as the concentration of the extract increases. It was found that at 200 µg/ml concentration of the extract showed 75% death of cancerous cells in the medium and 65% death at 100 µg/ml concentration (Table 5). However, it was also reported that the crude aqueous extract of the leaf exerts antiproliferative effects in A549 lung cells by increasing oxidative stress, DNA fragmentation and inducing apoptosis (35). The morphological changes of the HeLa cells treated with various concentrations of *M. oleifera* extract were typical of apoptosis and proposed the involvement of mitochondria also (36). Hence, we conducted molecular docking studies with emodin-8-glucoside with anti-apoptotic as well as signaling proteins (*vide infra*).

LCMS-QTOF analysis and molecular docking study

Compounds like Emodin 8-glucoside, His Ser His, 11-amino-undecanoic acid, GPEtn (20:0/17:0)[U], GPEtn (22:0/26:0)[U], 1-hexadecanoyl-2-(9Zhexadecenoyl), 3octadecanoylsn-glycerol, TG(16:1(9Z)/20:0/20:3(8Z, 11Z,14Z))[iso6], 1-heptadecanoyl-(9Z,12Zheptadecadienoyl)-3-octadecanoyl-sn-glycerol, 1-hexadecanoyl-2-(11Z-octadecenoyl)-sn-glycerol and TG(17:1(9Z)/17:2(9Z,12Z)/22:3(10Z,13Z,16Z))[iso6] were detected from lyophilized aqueous extract of *M. oleifera* leaf (Table 7). Emodin-8-glucoside is an anthraquinone and has got a striking chemical resemblance to anthracyclines, which are clinically well-established anticancer drugs (10-12) and since no reports of anti-proliferative activity of emodin-8-glucoside from *M. oleifera* was reported, it was further studied here by *in silico* method.

Molecular docking analysis was done to state emodin-8-glucosides could be one among the other several components present in the extract showing anti-cancer property. There are also reports that

suggest that aloe - emodin can be a potential candidate for anti-cancer therapy (37). Recently it was also reported that the emodin inhibits human cancer cell invasiveness by antagonizing P2X7 receptor (38). Emodin-8-glucoside was docked against proteins involved in crucial cell signalling processes such as AKT1, NF-kB (p65), Stat3 and anti-apoptotic proteins such as Bcl-2, Bcl-xl and c-Flip (Table 8, Fig. 3a-f).

AKT also called protein kinase B (PKB), is a member of PI3K/AKT signalling pathway. AKT isoforms (AKT1, AKT2 and AKT3) are often upregulated in cancer cells (39). AKT1 is ubiquitously expressed and directly responsible for growth factor mediated cell survival, prevention of apoptosis by inactivating pro-apoptotic proteins and angiogenesis (40–42). Importantly, tumour suppressor protein p53 is also regulated by AKT signalling and hence the oncogenic activation of p53 through AKT results in rapid destabilization of p53 and results in antiapoptotic signal (43, 44). Hence, AKT is an active target for anti-cancer therapy and AKT inhibitors were found to supress cancer progression. In this study, emodin-8-glucoside showed high affinity to bind with AKT1 with a binding energy of -10.5kcal/mol, showing its potential interactions with AKT1, possibly leading to its inactivation. In this binding of emodin-8-gludoside with AKT-1, van der Waal's interactions were formed with Gln-79, Tyr-272 and Ile-290, conventional hydrogen bond with Thr-211, carbon-hydrogen bond with Ser-205, pi-anion interaction with Asp-292, Pi-Pi stacked interaction with Trp-80, alkyl and Pi-alkyl interactions were with Leu-264, Lys-268 and Val-270 and Leu-210 (Table 8, Fig. 3).

NF-*k*B transcription factor family consists of five subunits; p65 (RelA) is one among them. NF-*k*B signalling pathway is involved in diverse cellular processes including cell proliferation and differentiation, immunity, stress, inflammation and apoptosis (45–48). Altered NF-*k*B pathways are often associated with different forms of malignancies and therefore NF-kB inhibition is an unquestionable target for cancer treatment. Also, several NF-kB inhibitors are not effective as an anti-cancer agent since its exact mechanism of action is unknown. Emodin-8-glucoside binds with p65 with a binding score of -7.3 kcal/mol. The interactions formed include van der Waal's interaction with Ser-261, Ala-264, Thr-292, Asp-293, Arg-297 and Glu-299, conventional hydrogen bonds Gln-263, Asp-291 and His-296, alkyl and pi-alkyl interactions were formed with Pro-265 and Pro-290 (Table 8, Fig. 3b).

Stat3 (signal transducer and activator of transcription3) is activated inappropriately and the downstream expression of target genes are the hallmark of many solid and haematological cancers. Untimely activation of the gene that are activated by stat3 signalling cascade are hallmarks of cancer (49). So, the inhibitors of stat3 are also possible targets for cancer drug research. Emodin-8-glucoside showed high affinity to bind with stat3 with a binding score of -7.6 kcal/mol and the interactions formed were of van der Waal's interaction with Gln-247, Ala-250, Cys-251, Ile-252, Gly-253, Glu-324, Arg-325, Gln-326, Cys-328, Arg-335, Pro-336, Trp-474, conventional hydrogen bond and Pi-anion interaction with Asp-334, carbon hydrogen bond with Ser-514, alkyl and pi-alkyl interactions with Pro-256. Unfavourable acceptor-acceptor interaction was there with Pro-333 (Table 8, Fig. 3c). Further studies may help to identify the possible mechanism of action.

The antiapoptotic proteins Bcl-2 and Bcl-xl function by preventing either the oligomerization of BAK-BAX by directly binding to BH3 domain of activator BH3 only proteins or they bind with activated BAK-BAX and induce conformational changes, thus preventing apoptosis (50–52). Bcl2 overexpression is also reported in many forms of leukaemia and lymphomas. Development of potent Bcl-2 antagonists as anti-apoptotic and anti-cancer agents are being investigated as therapeutic approach to treat cancer (53-55). Emodin-8-glucoside is a natural compound from *M. oleifera* leaf having a binding score of -7.2 kcal/mol with Bcl-2 and -8.3 kcal/mol with Bcl-xl, and showing high potential to bind with these anti-apoptotic proteins. This compound targets both Bcl-2 and Bcl-xl hence, it is an important lead compound for anti-cancer therapy. Following interactions were formed between emodin-8-glucoside and Bcl-2; van der Waal's interaction with Asp-111, Met-115, Glu-136, Arg-139, Asn-143, and Phe-153, conventional hydrogen bond was formed with Asp-140, pi-sigma with Phe-112, Pi-Pi T-shaped interaction with Tyr-108 and Phe-104 and Pi-alkyl interaction was with Ala-149 (Table 8, Fig. 3d). With Bcl-xl, emodin-8-glucoside formed van der Waal's interaction at Phe-97, Asp-107, Phe-131, Asp-133 and Phe-146, conventional hydrogen bonds were formed with Leu-130 and Arg-139, carbon-hydrogen bond and Pi-anion interaction with Glu-129, alkyl and pi-alkyl interactions were with Ala-104, Phe-105, Leu-108, Ala-142 and Ala-149 (Table 8, Fig. 3e).

c-FLIP is a cellular FLICE like inhibitory protein and is an anti-apoptotic protein which inhibits TNF-α, Fas-L and TNF-related apoptosis inducing ligand (TRAIL) induced apoptosis and also chemotherapy induced apoptosis in malignant cells. Binding of cFLIP to caspase-8 or -10 prevents the formation of Death Inducing Signalling Complex (DISC) and activation of caspase cascade (56). Upregulation of c-FLIP is often associated with various forms of malignancies (57). In the present *in-silico* study, emodin-8-glucoside showed high affinity to bind with cFLIP with a binding score of -7.4 kcal/mol. Binding of emodin-8-glucoside with c-FLIP resulted in the formation of van der Waal's interaction with Ser-318,Val-362, Ser-363, Thr-407, Ala-408, Met-410, Lys-460, and Lys-462, Pi-Pi stacked interaction with Tyr-464, alkyl and pi-alkyl interactions with Trp-466 and Leu 405. Unfavourable acceptor-acceptor interaction was also noted with ala-459 and Asp-409 (Table 8, Fig. 3f).

An inhibitor with potential for binding with several anti-cancer targets are always the most promising compound for anti-cancer therapy (58). Emodin-8-glucoside is one such compound obtained from *M. oleifera* leaf aqueous extract showing binding affinity with AKT1, NF-kB (p65), Stat3, Bcl-2, Bcl-xl and cFLIP in the present docking study. Thus, it

is a compound capable to bind with multiple cancer targets. Based on our results and available literature, the anti-cancer property of *M. oleifera* leaf water extract is possibly due to the presence of emodin-8-glucoside compound. The compound attracts further attention towards unravelling its novel action and possible mechanism in its purified form, apart from crude extract as a potent antineoplastic drug.

Conclusion

Moringa oleifera leaf lyophilized aqueous extract contained bioactive components, which showed potent antioxidant property *in-vitro* and *in-vivo*, and anti-cancer property *in-vitro* and *in-silico*. With the support of our *in-vitro* and *in-silico* results, we report emodin-8-glucoside as one of the bioactive components in the Moringa leaf water extract with potent anti-proliferative activity. *M. oleifera leaves* can be a nutritive diet component to prevent cancer in the light of our observation and various its other established pharmacological actions.

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Authors' contributions

AND carried out the analysis, conducted molecular docking studies, statistical analysis and drafted the manuscript. TJJ and SSL designed and coordinated the work and finalized the manuscript. BJK provided the infrastructure to conduct the animal study and supervised the same.

Conflict of interests

Authors do not have any conflict of interest to declare.

Ethical issues

All the study protocols were carried out as per CPCSEA and IAEC norms (Order No. Acad(3)/6554/04, dated 27/09/18).

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