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In vitro anti-oxidant activity of phytochemical analysis and various human cancerous cell line using microtiter plate based assay *Moringa oleifera* leaf extract

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Abstract
Moringa oleifera leaves, bioactive compounds within are may possess cancer-selective ant proliferative properties. Past research has been directed with respect to this subject, however poor trial configuration because of absence of vital controls restricts the authenticity of anticancer cases. *Moringa oleifera* plant contains a numerous antioxidants, antibiotics and nutrients (vitamins and minerals) which makes it prospective for diverse biomedical applications. *In vitro* antioxidant. This mostly contributes to the antioxidant power of the extracts, which is affirmed by the IC50 values of the crude extracts in the *Moringa oleifera*. (A) DPPH scavenging activity (B) H_2O_2 scavenging activity (C) Nitric oxide scavenging activity (D) FRAP assay. The FRAP assay also exhibit a constant increase in reducing ability with increase in the concentration which is indicative of the extract's antioxidant potential. In order for anticancer claims to be sufficient and yield the possibility of a future cancer treatment, *Moringa oleifera* leaves extract must not harm non-cancerous cells. The prevention and treatment of a series of chronic diseases including inflammatory diseases, neuro-dysfunctional diseases, diabetes, and cancers which will provide a reference for its possible application in the prevention and treatment of chronic diseases or health encouragement. The present study suggests that the hydro-alcoholic leaf extract of *M. oleifera* induces anticancer effect on K-562, DU-145 and HCT-15 cancer cells.

Keywords: *Moringa oleifera*, *In vitro* antioxidant, phytochemical, DNA fragmentation

1. Introduction
Ayurvedic medicine, one of the world's oldest holistic healing systems, claims that *Moringa* can prevent up to 300 diseases, and sideways from preventative measures, its leaves are capable of curative properties as well [1]. The traditional uses of *Moringa* are great in number, for they include the treatment of bacterial, fungal, viral, and parasitic issues, along with asthma, circulatory, digestive, and inflammatory disorders [2]. Other targeted ailments include malaria, typhoid fever, arthritis, hypertension, and diabetes [3]. Because of *Moringa's* ability to improve the immune system, treatment of HIV and AIDS symptoms is also possible. *M. oleifera* is rich in a wide range of secondary metabolites including proteins, vitamins, b-carotene, amino acids and various phenolics as flavonoids and phenolic acids Medicinally, various parts of *M. oleifera* have been widely employed as cardiac and circulatory stimulants, antitumor, antiepileptic, diuretic, antihypertensive, cholesterol lowering, hepatoprotective, antioxidant, antibacterial and antifungal agents [4-5]. With this vast list of traditional medicinal properties, it comes as no surprise that *Moringa* is packed with chemical components to give it an astounding phytochemistry [6-7]. His release of material will lead to an inflammatory response by immune cells, which could possibly lead to further tumor growth.

2. Material Methods
2.1: Plant Collection
The fresh leaves of *Moringa oleifera* were collected from Saliyamangalam, Thanjavur District, Tamil Nadu, and India.

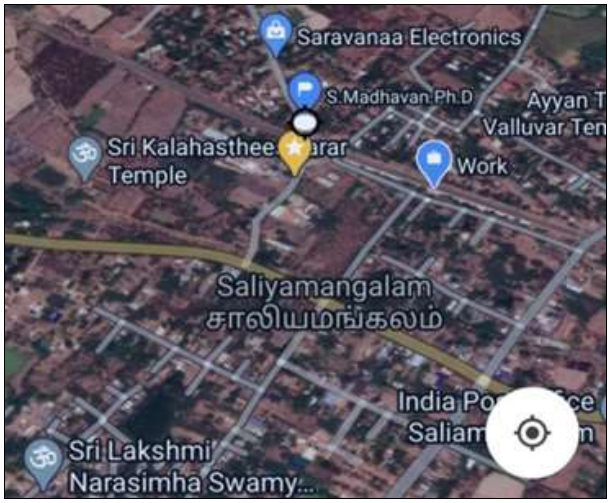

Fig 1: Map 1: Study area

Fig 2: *Moringa oleifera* leaf

2.2: Plant material
The *Moringa oleifera* leaf was dried up under shade, crude powder. The crude type of the medication was utilize for the declaration of physicochemical boundaries similar to dampness content, debris esteems, increasing case, frothing evidence, unfamiliar natural issue, extractive qualities, and fluorescence analysis.

2.3. Phytochemical Studies
Moringa oleifera Secondary metabolites in the present studies were presence of medicinally active constituents. Beneficial drugs and to improve the patient health.

2.4. Preparation of extracts
The powdered plant samples of leaves (100 g) were used for successive solvent extraction (500 ml) with increasing order of polarities like ethanol, methanol. At that point it is kept in an orbital shaker at 190-220rpm for 48 hours. The supernatant was collected, filtered through Whatman No.1filter paper and the extract were concentrated by a Rotary flask evaporator at a specific temperature was used based on the solvent system. Each time previous to extract through the next solvent the remains was dried thoroughly to remove the solvent used. The acquire dried up concentrate was then specifically gauged, put away in little vials at – 20 °C and utilized for the supplementary examinations.

2. 5. Phytochemical screening
The preliminary phytochemical evaluation was carried out by using standard procedure [8].

2.6. In-vitro anti-oxidant assays
2.7. DPPH free radical scavenging assay
The DPPH radical-scavenging activity of the test extracts was examined using the modified method by Brand-Williams *et al*. [9]. Leaf extracts of different concentrations (50e200 mg/mL) were mixed with an equal volume of methanolic solution of DPPH (Sigma Aldrich). The mixture was allowed to react at room temperature in dark for 30 min. Ascorbic acid (1 mg/mL (50e200 mg/mL)) was used as positive control. After 30 min the absorbance was measured at 517 nm and converted into percentage of antioxidant activity using the following equation.

% of inhibition = [A0-A1/A0] *100

Where
A0 =Absorbance of control.
A1 = Absorbance of test.

2.8. Hydrogen peroxide scavenging assay
The H2O2 scavenging activities for both the leaf extracts were assayed by the modified method [10]. Different concentrations of plant leaf extracts (50e200 mg/mL) and ascorbic acid at different concentrations (50e200 mg/mL) of (1 mg/mL) were added to 40 mM H2O2 solution prepared in phosphate buffer. The absorbance of H2O2 at 230 nm was determined after 10 min. The percentage of H2O2 scavenging by the extracts and standard (H2O2) was calculated as follows.

% of scavenged [H2O2] = [A0-A1/A0] *100

Where
A0 =Absorbance of control.
A1 = Absorbance of test

2.9. Nitric oxide radical scavenging assay
The nitric oxide (NO) scavenging activity was determined using the method described by Parul *et al*. [11]. 10 mM sodium nitroprusside was incubated with 100 mL leaf extract for 60 min at 30 _C. After incubation, 100 mL of griess reagent was added. The absorbance of the chromatophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylehylendiamine was measured at 562 nm. Ascorbic acid (1 mg/mL) was at the same concentration was taken as standard.

% NO scavenged = [A0-A1/A0] *100

Where
A0 =Absorbance of control.
A1 =Absorbance of test.

2.10. Ferric reducing power (FRAP) assay
The reducing power was determined by Benzie and Strain [12] with slight modifications. Various concentrations of plant leaf extracts (50e200 mg/mL) were mixed with phosphate buffer and 2 mM potassium ferricyanide. The mixture was incubated at 50 _C for 20 min. TCA was added

to the mixture which was then centrifuged at 3000 rpm for 10 min. The upper layer of solution was mixed with distilled water and freshly prepared Fecl3 solution (0.5 mL) and the absorbance was recorded at 700 nm using UV-Visible spectrophotometer (Thermo scientific evolution -201 series). Ascorbic acid (50e200 mg/mL) was used as positive control. Reducing capacity was calculated as follows:

% increase in reducing power = [Atest/Ablank-1]*100

Where
Atest =Absorbance of test solution.
Ablank = Absorbance of blank.

2.11. Culturing of cell lines
The Vero and human cellular breakdown in the lungs cells (HCT-15, MCF-7, HEP-3B, K-562 and DU-145 and VERO) were acquired from Kings Institute of Preventive Medicine and Research, Guindy, Chennai. The cells were grown in 96 well tissue culture (TC) plate in Dulbecco's Minimum Essential Medium (MEM) with Trypsin-phosphate-verseneglucose (TPVG) solution, 10% New Born Calf Serum (NBCS) (Gibco-Invitrogen), 100 U/mL of penicillin (Gibco-Invitrogen) and 100 μg/mL of streptomycin (Gibco-Invitrogen). The cells were incubated in CO_2 incubator (Haier Electric Co., Ltd.,) at 37oC in 95% humidified atmosphere enriched by 5% CO_2 and sub-cultured every 3-4 days once.

2.12. Cell Proliferation Assay (MTT Assay)
The cytoxicity study of the methanolic and ether extracts of leaves and root of plant was studied on cultured cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (29). Cells were seeded in 96-well plates in DMEM supplemented with 10% FBS. After 24h of incubation in a humidified 5% CO2/air environment at 37 ^{o}C, when cells became 70-80% confluent, then the cells were grown in 96-well plates for establishment of monolayer at a density of 1×105 cells per well according to the instruction given in kit manual (EZ count MTT Cell Assay Kit, PC:CCK003, Hi-Media). After achieving desired cell density, cells were treated with one third dilution series of various extract concentrations (0.05, 0.15, 0.46, 1.37, 4.12, 12.35, 37.04, 111.11, 333.33 and 1000µg/ml) and incubated further for 24 hrs. Then after next day, 25µl of the MTT solution (5mg/ ml) was added to each well, and the plate was re-incubated for 4hrs. Finally, 100µl of DMSO: IPA (60:40) solubilizing mixture was added to dissolve formazan crystals. Then absorbance of the plate was measure at 570nm by using a 96 well micro plate reader (Multiskan Reader CF3, Thermo scientific). Percentage cell growth inhibition or percentage cytotoxicity was calculated by following formula:

%Viability = (AT-AB)/(AC-AB)×100

Where,
AT=Absorbance of treated cells (drug), AB=Absorbance of blank (only media) and AC=Absorbance of control (untreated).

2.13. DNA fragmentation study
The DNA fragmentation analysis was carried out to distinguish the apoptosis from necrosis, and is among the most reliable methods for detection of apoptotic cells. The DNA fragmentation study was performed by selecting the IC50 dose of the various extracts of *Moringa oleifera* against cancer cell lines K-562, DU-145. Standard kit manual protocol (Apoptotic DNA Ladder Kit, KH01021, Thermo fisher, USA) was followed to perform DNA fragmentation study.

Microscopy
To monitor cell morphology, cells were visualized by light microscopy (Leica Microsystems, DMIL, and Germany). Images were captured with a Power Shot S45 Canon Digital Camera system.

Statistical analysis
All assays were performed in triplicate. Mean and standard deviation (SD) was examined for all assays. The results were expressed as mean ± SEM of three experiments. One way ANOVA with Dunnett's test was followed to compare each concentration with positive control to analyze level of statistical significance. $P < 0.05$ were considered statistically significant using Graph pad PRISM v.8.0.

3. Results and Discussion
3.1. Preliminary phytochemical screening
The consequence of the starter phytochemical examination of this current investigation may offer assurance to its ethnomedicinal utilization [13]. The free revolutionaries present in our body are answerable for the age of numerous sicknesses [14]. In medication, it is utilized in hypercholesterolemia, hyperglycaemia, cell reinforcement, anticancer, calming, and weight reduction among others. It is likewise known to have antimicrobial properties. India is in all probability the best maker of remedial flavors on the planet.

Table 1: Qualitative analysis of Phytochemicals analysis *Moringa oleifera leaves* extract

S. No	Analysed Phytochemicals factor	Aqueous	Methanolic
1.	Tannin	+	+
2.	Saponin	-	+
3.	Flavonoids	+	+
4.	Steroids	+	-
5.	Alkaloids	+	-
6.	Polyphenol	+	+

Indications: "+" means positive activity, "-" means negative activity

Each constituent assumes a significant part and lack of any one constituent may prompt unusual advancements in the body [15]. While previous research has shown that *Moringa* leaf extract has the potential to kill cancer cells, the research fails to demonstrate the effects of *Moringa* leaf extract on healthy cells. The current investigation express that the presence of methyl or ethyl esters of unsaturated fats can likewise be considered as qualities of this plant. From this outcome, it very well may be reasoned that every one of these mixes are of pharmacological significance as they have the properties, for example, antibacterial enemy of diabetic and pain relieving.

3.2. *In-vitro* anti-oxidant
Macrophage response to microbes is almost as fast as

neutrophils, but macrophages live longer than neutrophils. Macrophage phagocytosis is also more active in dealing with pathogens such as microorganisms or other antigens, and even cells or tissues themselves are damaged or dead so that macrophages can be categorized as primary effector cells in the natural immune response.

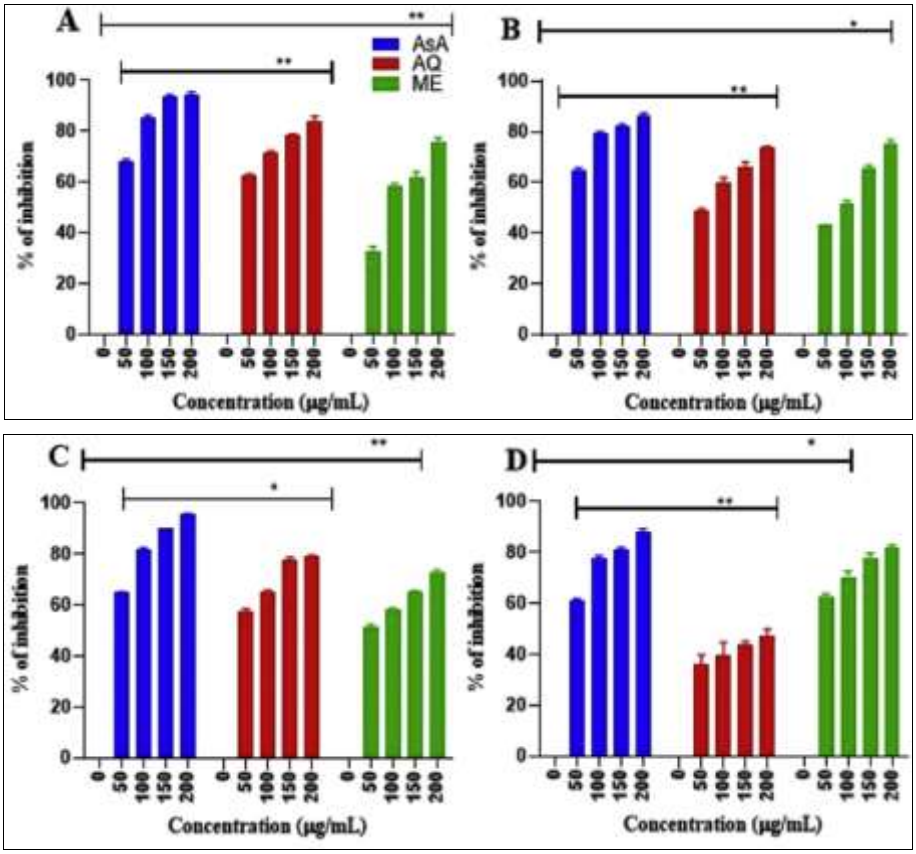

Fig 3: In-vitro antioxidant activity of aqueous and methanolic leaf extracts of *Moringa oleifera*. (A) DPPH scavenging activity (B) H_2O_2 scavenging activity (C) Nitric oxide scavenging activity (D) FRAP assay. Values are expressed as Mean ± SEM (n ¼ 3). One-way ANOVA followed by Dunnett's test was employed to compare each concentration with positive control. *Statistical significance at $p < 0.05$; ** statistical significance at $p < 0.01$. AsA-Ascorbic acid (Positive control); AQ - Aqueous; ME-MeOH.

3.3 Anticancer Activity

MTT is divided by all living, metabolically dynamic cells that we have tried, yet not by dead cells. In the current work, the concentrate was considered in contrast to chosen cell lines named K-562, DU-145, MCF-7, HCT-15, HEP-3B and VERO cell line (Ordinary cell). For each tried compound, Portion Reaction Bend (DRC) against all cell lines was plotted with 10 examination focuses for example with 10 diverse medication fixations. This measure was additionally performed for Colchicine as standard compound against same cell lines. Result for every cell lines.

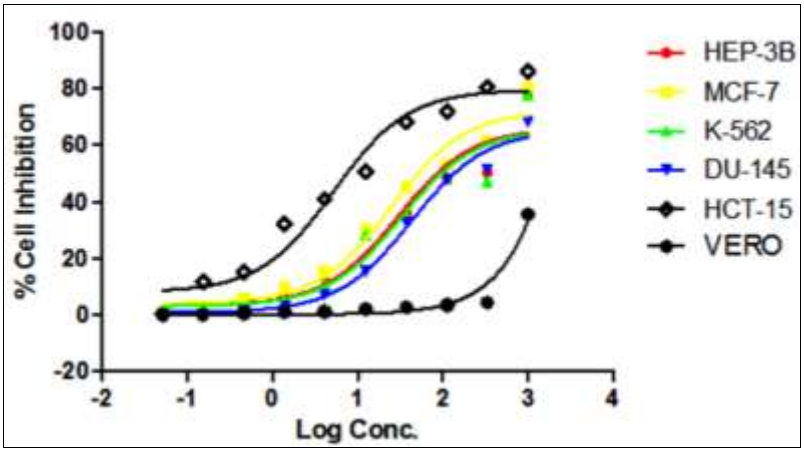

Fig 4: Dose Response Curve of hydro-ethanolic extract of *Moringa oleifera* leaves on various cell lines.

In vitro cytotoxicity study of K-562, DU-145, HCT-15, MCF-7, HEP-3B and VERO cell lines for hydro-alcoholic extract. It showed order of cytotoxicity activity in comparision to standard Colchicin drug (Table 1). Potency of the extract for cell inhibition obtained was K-562(IC50 value 32.43µg/ml, R2=0.9253)>DU-145(IC50 value

42.74µg/ml, R2=0.9862)>HCT-15(IC50 value 5.213µg/ml, R2=0.9647))>MCF- 7(IC50 value 24.76µg/ml, R2=0.9701))>HEP-3B (IC50 value 29.37µg/ml, R2=0.9377). *Moringa oleifera* leaves extract showed good cytotoxicity activity against K-562 cell line while, same induced less activity in MCF-7 and HEP-3B. Dose Response Curve of hydro-ethanolic extract of *Moringa oleifera* leaves on various cell lines.

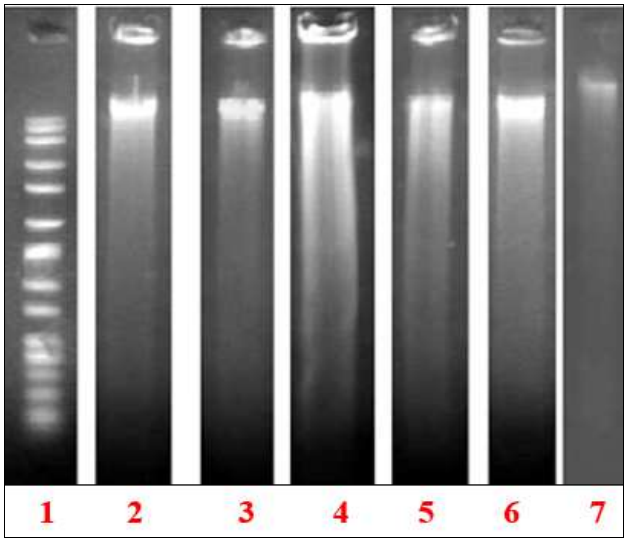

Fig 5: DNA fragmentation study on different cancer cell lines. Lane 1: 100 BP DNA ladder; Lane 2: HEP-3B cell line; Lane 3: MCF-7 cell line; Lane 4: K-562 cell line; Lane 5: DU-145; Lane 6: HCT-15 cell line; Lane 7: VERO cell line

DNA fragmentation study was carried out from the results of DNA fragmentation, it was found that the extract was exhibited excellent DNA fragmentation pattern in K-562, DU-145 and HCT-15 comparison with standard DNA ladder, which confirms the apoptosis mechanism rather necrosis properties. In addition, less DNA fragmentation pattern was observed in MCF-7 and HEP-3B indicated less cytotoxicity effect. The possible reason behind cytotoxicity activity is because of the leaves contains most of nitrile glycoside derivatives. But further study is required to confirm the same for its molecular mechanisms by studying various cancer pathways

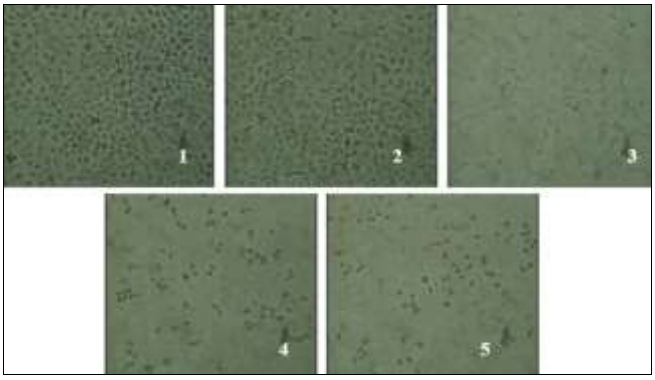

Fig 6: (1) MCF-7 cell (2) HEP-3B cell (3) K-562 (4) HCT-15 cell (5) DU-145 cell

Home grown intervened silver nanoparticles assuming significant part to make eco-accommodating, savvy and stable nanoparticles. The researches on association of silver nanoparticles using distinctive plant eliminates found that it is safer and better in dangerous development treatment, anyway more plants are at this point not examined for the blend of nanoparticles and its applications in drug organizations. By and large chemotherapy, activity and radiation treatment are the most notable helpful alternative for perilous improvement sadly; these medications have different reactions because of nonappearance of focused vehicle [16]. Combination of home grown intervened silver nanoparticles gives controlled and focusing on activity of medication, which can likewise defeat the issues related with traditional disease medicines.

4. Summary and Conclusion

Secondary metabolites phytochemical analysis presence of absence *In vitro* antioxidant, anti-cancer and anticoagulant activities to scientifically validate their folklore use in treatment of diseases [17]. This is a first- hand report that provides sufficient evidence for carrying out further research on the selected plants to decipher the exact mechanism involved in anticancer and anticoagulant activity. Thereby suggesting *in vitro*, *in vivo* and secondary metabolite profiling studies to unravel and identify the bioactive compounds responsible, and ultimately provide alternative treatment strategies. Clinical oncologist utilize chemotherapeutic specialists that regularly effectsly affect organs regardless of their viability against malignancy cells. The adequacy of these medications is restricted bringing about administration of unfriendly medication responses, opposition and conceivable treatment-related demise [18-19]. Pharmacodynamics and pharmacokinetics assume an essential part for fruitful patient results. In this manner, it is basic to evaluate during the plan and advancement phase of elective medications. We have proposed the conceivable component for poly phenolic collaboration with gold metal particles for the phyto-nanoparticles development. To distinguish the decreasing specialists present in the concentrate which caused the decrease Malignancy is delegated one of the main sources of worldwide mortality. It has influenced a large number of individuals, regularly with helpless guess. Having serious results with customary chemotherapy, substitute medications and treatments are effectively being examined. There is a requirement for inventive medication disclosure and plan as existing malignant growth treatments are exorbitant and not promptly accessible. The *Moringa oleifera* methanolic extricate showed the anticancer movement against A549 cell line in a portion dependant manner.A549 cells treated *Moringa oleifera* methanolic remove in various fixation level (100, 200, 250, 500 and 1000 µg/ml) after the 36 hours the phones development are changes happened. Along these lines, more examination is needed to associate its pharmacological action with science based confirmations by strengthing linkage between investigates being done by various gatherings over the world so it tends to be created as expected medications. The current investigation can be considered as a primary investigation paving the way for future work investigating the possible mechanisms and modes of action on various human cancerous cell lines. Results of anticancer activity study showed that extract of leaves induced severe cell cytotoxicity in K-562 and DU-145 cancer cells; however it was not the case anymore in normal cells. Further confirmation for anticancer activity of extract using DNA fragmentation assay demonstrated good DNA fragmentation properties in leukemic and prostate cancer cells, which indicates good apoptosis effect. Overall,

these data suggest that the hydro-alcoholic leaves extract of *Moringa oleifera* may become a good candidate for anticancer therapy in leukemia and prostate cancer. Further studies are required in this regard. Ayurveda and traditional medicine have utilised natural resources such as plants and vegetation as part of their government to treat various illness and diseases with positive outcomes. One such tree is *Moringa oleifera*. In addition, these natural medicinal plants can be used for the synthesis of phyto-nanoparticles with targeted anticancer properties. Advancing medicine these agents can provide a source of easily accessible and affordable therapies in the future.

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6. Conflict of Interest
The authors have declared that there is no conflict of interest.

7. Source/S of Funding
No source of funding.

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