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THE ANTIANGIOGENIC, ANTIOXIDANT AND PROAPOPTOTIC CHEMOPREVENTIVE PROPERTIES OF TANNINS FROM *MEMECYLON MALABARICUM* (Cl.)

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ABSTRACT: Tannins, the non-nutritive substances from plant sources exhibit potent biological activities that lower the risk of chronic diseases without any side effects. Tannins are most abundant and multipotent molecules exhibiting antioxidant, proapoptotic and antiangiogenic activity and thus being effective in the treatment of degenerative diseases including initiation and progression of tumors. The study aims to prove the chemopreventive properties of tannins that target several key events involved in the development of cancer. Partially purified tannins from the acetone extract of *Memecylon malabaricum* was subjected to shell less CAM assay and in vitro studies to note the antiangiogenic and antioxidant activities respectively, while the proapoptotic activity was noted by its effect on EAT cells. Inhibiting the proliferation of capillaries in shell less CAM assay proved their antiangiogenic property. 1, 1-diphenyl 2-picrylhydrazyl (DPPH) radical scavenging activity (EC₅₀ of 2.67 µg/ml), Hydroxyl radical scavenging activity (EC₅₀ of 7.73 µg/ml), Nitric oxide radical scavenging activity (EC₅₀ of 11.3 µg/ml) and superoxide anion radical scavenging activity (EC₅₀ of 19 µg/ml) established their antioxidant activity, while the proapoptotic activity of the compound on EAT cells showed characteristic nuclear condensation with cytoplasmic and nuclear blebbing. The induction of apoptosis is attributed to DNA fragmentation incited by tannins. The leaves of *MM* which are primarily rich in a wide group of natural multipotent molecules like tannins with manifold chemopreventive activities represent a promising class as anticancer drugs.

INTRODUCTION: The central process of angiogenesis involves the development of new blood vessels from the pre-existing vessels in pathological and physiological conditions. Tumor progression predominantly involves the development of new blood vessels. However recent research reveals that mechanisms of tumor growth is associated with vasculogenic mimicry, vessel cooption, lymphangiogenesis, intussusceptive angiogenesis and the recruitment of endothelial progenitor cells along with the existing mechanisms like angiogenesis and vasculogenesis¹.

Hydroxyl radical (HO[·]), superoxide anion radical (O₂^{·-}), peroxy radical (ROO[·]), alkoxy radical (RO[·]), alkyl radical (R[·]) and nitric oxide radical (NO[·]) are the frequently encountered free radicals in the biological system.

These are generated endogenously and even by adverse environmental factors and attribute for the various destructive diseases including tumor initiation and progression². Antioxidant, anti-angiogenesis, and pro-apoptotic activity represent important activities for preventing, suppressing, or reversing the development of tumor progression. Majority of the therapeutic drugs against cancer potentially target any one of these mechanisms which occur parallel and also provide useful basis for screening novel phytochemical compounds for chemo preventive activity³.

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Cytotoxic drugs induce death of cancer cells by apoptosis. Apoptotic cells are morphologically characterized by nuclear condensation with or without the fragmentation of DNA, cellular shrinkage, relocalisation of cell organelles and blebbing of nuclear and plasma membrane⁴. Apoptotic bodies are the blebs formed on the surface of the cell by hyper segmented nuclear structure⁵. A promising strategy for the treatment of cancer involves inhibition of angiogenesis and induction of apoptosis⁶.

Many of the phytochemical compounds exert various pharmacological and physiological effects, like stimulating and strengthening the immune system, protecting the heart from free radical attack and inactivating cancer causing substances⁷. These chemo preventive properties are attributed to the potent biological activities shown by few of the non-nutritive antioxidant substances like polyphenols or tannins that lower the risk of chronic diseases without any side effects⁸.

Hence, there is a renewed interest in phytochemicals to prevent various degenerative diseases. Tannins are most abundant and potent biological antioxidants that widely exhibit many biologically important functions which include protection against oxidative stress, and degenerative diseases including initiation and progression of tumors. Recent work suggests that tannins could be free form, protein-complexed or condensed having molecular weights between 500 - 3000KDa and are more effective than the small phenolic compounds.

Besides giving the usual phenolic reactions, they possess special properties such as the ability to precipitate alkaloids, gelatin and other proteins⁹. There is an increasing body of experimental evidence validating the possible anti-initiating, anti promoting and chemo protective activity of the tannins against cancer in a number of animal models.

As a first step towards identification of phytochemical potential for antitumor and chemopreventive activity we have targeted evaluation of tannins from the leaves of *MM*, which is abundantly used in ayurveda and traditional medicine to cure many ailments with their

antioxidant, antiangiogenic and pro-apoptotic activity.

MATERIALS AND METHODS: *Memecylon malabaricum* leaves were collected from Western Ghats, Karnataka state, India. The plant was identified and authenticated by a taxonomist. All chemicals were of analytical grade.

Isolation purification and identification of tannins: The leaves of *MM* were separated, washed, shade dried ground to powder, sieved and stored in air tight container. 100 gm of dry powder of the leaves of *MM* was taken in soxhlet apparatus and subjected for sequential extraction of solvents from non polar to polar end using hexane, chloroform, ethyl acetate, acetone, and methanol. Acetone extract was treated with 10% (wt/v) activated carbon and filtered. The filtrate was treated with toluene and centrifuged at 1500 rpm for 10 min; supernatant which was rich in chlorophyll was discarded. The above step was repeated twice to remove all the chlorophyll.

The residue was treated with isopropanol, centrifuged at 1500 rpm for 10 minutes and the supernatant was discarded, the residue was again dissolved in methanol and was loaded on activated silica gel TLC plates (20X20 cm). The plates were developed using the solvent system methanol: acetic acid (90:10). The bottom most brown coloured band was scraped and dissolved in methanol and filtered.

The compound was tested for its purity by HPLC using a LC-10AT liquid chromatography (M/s Shimadzu, Singapore) equipped with a C-18 column (Prevail C-18, 5 μ m, 150 mm from M/s Alltech, Germany; fitted with a refillable guard column from M/s Waters India Ltd., India) and methanol: water: acetic acid (85:35:10) as a mobile phase with a flow rate of 1.5 ml/min. Ultraviolet (UV) detection was carried out with a diode array detector (M/s Shimadzu, Singapore). The detector was operated at an UV wavelength detection of 280 nm.

Shell less chorioallantoic membrane (CAM) assay: Antiangiogenic effect of the tannins was studied according to the method of Auerbach et al¹⁰. Briefly, fertilized hens eggs were surface

sterilized using 70% alcohol. The eggs were incubated in fan assisted humidified incubator at 37°C. On the 4th day, the eggs were cracked out into thin films of the hammock within a laminar flow cabinet and were further incubated.

On the day 5th when blood vessels were seen proliferating from the centre of the eggs within the hammock, filter paper discs loaded with 100 µg of tannins from *MM* were placed over the proliferating blood vessels and the eggs were returned to the incubator. Results for antiangiogenic effect of the compound were observed after 24 hours.

DPPH radical scavenging assay: DPPH radical scavenging activity was carried out according to Scherer R et al. method¹¹. Briefly, 1ml of DPPH solution (0.1mM in 95% ethanol) was mixed with different aliquots of 1- 5µg of the tannin sample. After vigorous shaking, the mixture was allowed to stand for 20 min at room temperature. Absorbance of the resulting solution was measured at 517 nm with a UV-VIS spectrophotometer (HITACHI, U-2900) Butylated hydroxyl toluene (BHT) was used as positive control. Radical scavenging potential was expressed as EC₅₀ value, which represents the sample concentration at which 50 % of the DPPH radicals were scavenged.

Hydroxyl radical scavenging assay: The reaction mixture containing different aliquots of 2-16µg of the tannins, deoxyribose (10mM), H₂O₂ (10mM), FeCl₃ (5mM), EDTA (1mM) and ascorbic acid (5mM) in potassium phosphate buffer (50mM, pH 7.4) was allowed to stand for 60 min at 37°C¹². The reaction was terminated by adding TCA (5% W/V) and the reaction product was measured by the reaction with TBA (0.2% W/V) in boiling water bath for 15 min. The absorbance was measured at 535nm against the reagent blank and inhibition of the oxidation of deoxyribose was calculated against the control using UV-VIS spectrophotometer (HITACHI, U-2900). BHT was used as positive control and radical scavenging potential was expressed as EC₅₀ value.

Nitric oxide radical scavenging activity: Nitric oxide was generated from sodium nitroprusside and measured by Griess reaction. Sodium nitroprusside in phosphate buffer at physiological pH

spontaneously generates nitric oxide, which in turn reacts with oxygen to produce nitrite ions that can be estimated by the Griess reagent¹³. Nitric oxide scavengers compete with oxygen, leading to reduced production of nitric oxide.

Sodium nitroprusside (5mM) in phosphate buffered saline was mixed with different aliquots of 2-22µg of tannins and incubated at 25°C for 3 hrs. The absorbance of the color formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine was read at 546 nm and referred to the absorbance of BHT treated in the same way with the Griess reagent. The radical scavenging potential was calculated and expressed as EC₅₀ value.

Superoxide radical scavenging assay: The superoxide radical scavenging ability of the isolated sample was measured according to Nishikimi et al. method¹⁴. The reaction mixture containing different aliquots of 5-25µg of tannins, PMS (0.1mM), NADH (1mM) and NBT (1mM) in phosphate buffer (0.1M, PH 7.4) was incubated at 28 °C for 5 minutes. The color reaction was recorded at 560 nm using UV-VIS spectrophotometer (HITACHI, U-2900). BHT was used as positive control and radical scavenging potential was expressed as EC₅₀ value.

Apoptotic morphology of EAT cells

Giensa and acridine orange – ethedum bromide staining: EAT bearing mice was sacrificed by cervical dislocation and the EAT cells were harvested. The cells were centrifuged at 3000 rpm for 5 minutes and the packed cells were diluted 1:6 times with Phosphate buffer saline. 2ml of the diluted cells were treated with the isolated tannin compound (100 µg) and incubated for 4hrs at 37°C. Untreated EAT cells served as controls.

At the end of 4 hours, the samples were centrifuged and smears were made from the cell pellet obtained, fixed with methanol-acetic acid (3:1) and the morphological features of the cells were observed using different stains. Batches of both test and control smears were stained with Giensa's stain and acridine orange - ethedum bromide stain that highlights the apoptotic morphology of the cells when observed under bright field microscope and florescent microscope respectively¹⁵.

DNA fragmentation assay: DNA was isolated from both tannin treated and untreated cells after the incubation of EAT cells with 100 μg of tannin for 4 hours, reactions were terminated using ice cold Hank's Balanced Salt Solution (HBSS) and the supernatant was discarded after centrifugation. Cells were lysed with 50mM Tris-HCl buffer, pH 8.0 and 0.5% SDS which was incubated for 30 min at 37 $^{\circ}\text{C}$. The cell lysate was subjected to 8 M potassium acetate precipitation and left for 1 h at 4 $^{\circ}\text{C}$. The supernatant was subjected to phenol: chloroform: isoamyl alcohol (25:24:1) extraction and chloroform extraction. DNA was precipitated by adding 1:2 volumes of ice-cold ethanol. The precipitated DNA was dissolved in 50 μl of TE buffer (pH 8.0). The DNA was digested with 20 $\mu\text{l}/\text{ml}$ of RNase at 37 $^{\circ}\text{C}$ for 1 hour. The DNA was quantified and equal concentration of DNA (50 μg) was resolved on 1.5% agarose gel, viewed under UV light and documented using Bio-RAD.

Statistical analysis: All values are expressed as the mean \pm SD done in quadruplicates of two independent experiments. Statistical analyses between the groups were performed by the unpaired Student's t-test. EC50 value was calculated using origin 8.5 software.

Results:

Identification of bioactive compound from MM leaf: 100 gm of dry leaf powder of *MM* yielded 15.76gm of dry acetone extract. The isolated compound was identified as tannins as the compound formed a colloidal solution with water, formed bluish black color on reaction with ferric chloride and precipitated gelatin protein and salts of copper and lead. The purity of tannins was confirmed by TLC which revealed a single spot and further by HPLC which showed a single peak as in **Fig. 1**. The yield of tannins was found to be 3.3 gm/100 gm of dried leaf powder.

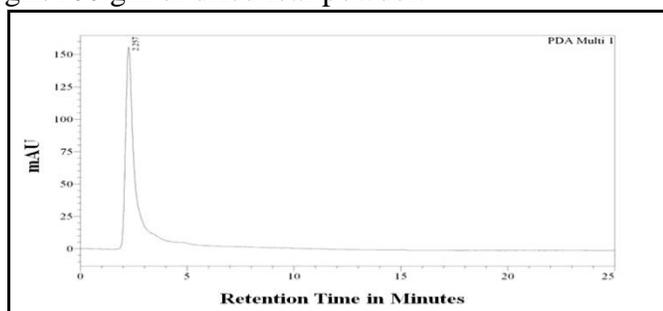


FIG.1: HPLC PROFILE OF THE ISOLATED TANNINS FROM *MM*. UV DETECTION AT 280 nm

Antiangiogenic activity of tannins from *MM*: Anti angiogenic treatment is one of the main methods of tumor treatment and control of pathological angiogenesis.¹⁶ Pathological angiogenesis is regulated by targeting integrins which are predominantly expressed in most of the tumor cells and the endothelial cells of blood vessels. Blocking of vascular endothelial growth factor (VEGF) and its receptors in signal transduction pathway also regulates angiogenesis¹⁷.

In the present investigation anti angiogenic activity of tannins from *MM* showed reduced proliferation of blood vessels in the shell less CAM assay model of developing embryos. The proliferation of microvessels regressed around the zone of tannin loaded disc as seen in **Fig. 2** compared to the control, thus supporting the antiangiogenic activity.

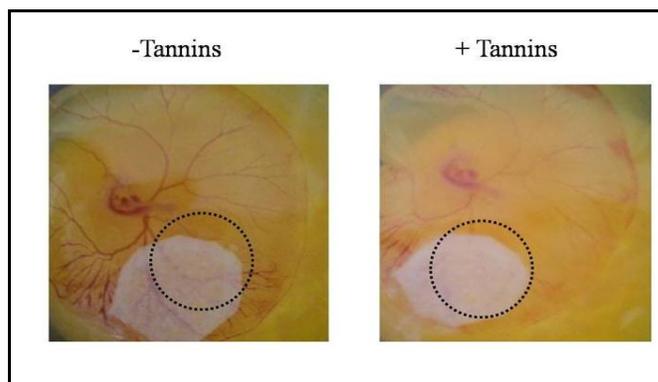


FIG. 2: INHIBITION OF ANGIOGENESIS IN TANNIN TREATED SHELL LESS CAM ASSAY

Antioxidant activity of tannins from *MM*

DPPH radical scavenging activity: Natural antioxidants are characterized by their ability to scavenge free radicals. Proton-radical scavenging action is an important attribute of antioxidants, which is measured by the DPPH scavenging assay. DPPH, a protonated radical has significant absorbance maxima at 517nm which decreases in the presence of antioxidant due to the scavenging of the proton radical¹⁸.

Hydrogen donating ability of the antioxidant molecule contributes to its free radical scavenging potential. The DPPH radical scavenging activity shown by the tannins is because of its H- donating capacity. In the present investigation tannins from leaves of *MM* showed high DPPH radical scavenging activity at the lowest concentration (2 to 3 μg) and are comparable with BHT. However,

increase in activity was marginal with increase in concentration as in **Fig. 3**. Tannins exhibited a high EC_{50} value of 2.67 $\mu\text{g/ml}$ when compare to that of the BHT which showed a value of 2.19 $\mu\text{g/ml}$.

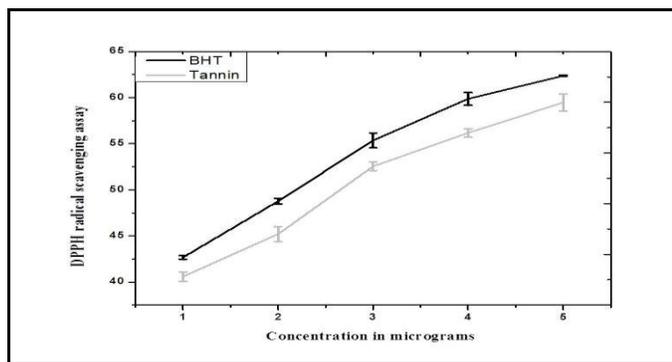


FIG. 3: DPPH ACTIVITY OF TANNINS (EC_{50} value 2.67 $\mu\text{g/ml}$) COMPARED TO BHT (EC_{50} value 2.19 $\mu\text{g/ml}$)

Hydroxyl radical scavenging activity: The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology and is capable of damaging biomolecules found in living cells¹⁹. Hydroxyl radical has the capacity to cause breakage of DNA, which results in cytotoxicity, carcinogenesis and mutagenesis.

In addition, this radical species is considered to be one of the quick initiators of the peroxidation of the lipid, removing hydrogen atoms from unsaturated fatty acids¹⁹. In this study the tannins isolated from *MM* showed high hydroxyl radical scavenging activity than standard BHT. The hydrogen donating capacity of tannins was observed to be much higher compared to BHT. The hydroxyl radical scavenging activity of tannins isolated from *MM* appears to be linear. Increased activity was observed with increasing concentration of tannins with an EC_{50} value of 7.73 $\mu\text{g/ml}$ as in **Fig. 4**.

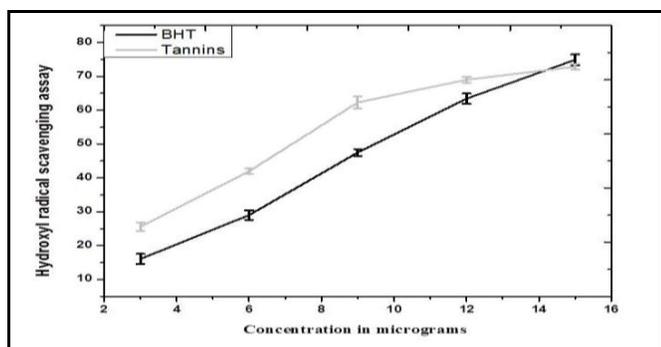


FIG. 4: HYDROXYL RADICAL SCAVENGING ACTIVITY OF TANNINS (EC_{50} value 7.73 $\mu\text{g/ml}$) COMPARED TO BHT (EC_{50} value of 10.10 $\mu\text{g/ml}$)

Nitric oxide radical scavenging activity: Under physiological condition, nitric oxide (NO) plays important role as a neurotransmitter, vasodilator and in the immunological system it fights against tumor cells and infectious agents.

During inflammatory reactions, NO is produced by the inducible enzyme NO synthase (iNOS) in cells like macrophages, hepatocytes and renal cells after the stimulation with lipopolysaccharide (LPS), tumor necrosis factor (TNF- α), interleukin (IL-1) or interferon (INF- γ) and acts as a defense and regulatory signal molecule. However, NO is pathogenic when present in excess. NO per se as a reactive radical, directly damages normal tissues²⁰. Further, nitric oxide can also react with superoxide anion radical to form an even stronger oxidant peroxy nitrite²¹.

The purified tannins from *MM* showed significant ($P < 0.01$) activity in scavenging nitric oxide radical assay with an EC_{50} value of 11.3 $\mu\text{g/ml}$ when compared to 15.2 $\mu\text{g/ml}$ recorded with BHT as in **Fig.5**.

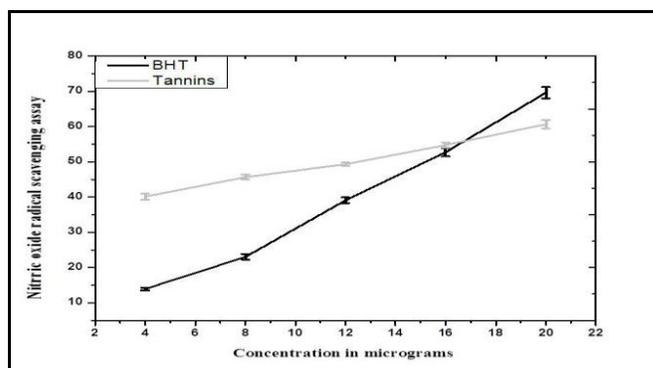


FIG.5: NITRIC OXIDE RADICAL SCAVENGING ACTIVITY OF TANNINS (EC_{50} value 11.3 $\mu\text{g/ml}$) COMPARED TO BHT (EC_{50} value 15.2 $\mu\text{g/ml}$)

Superoxide anion radical scavenging activity: The superoxide anion is produced in cells during the course of normal metabolism which is removed by enzymatic detoxification. Excessive production of superoxide in cells does occur due to both endogenous and exogenous factors.

Although superoxide anion is by itself a weak oxidant, it gives rise to the powerful and dangerous hydroxyl radicals as well as the singlet oxygen both of which contribute to the oxidative stress²². Therefore superoxide radical scavenging by antioxidants has physiological implications. The

purified tannins from the *MM* showed moderate activity in scavenging super oxide anion radicals compare to that of the BHT with an EC₅₀ value of 19.0µg/ml and 13µg/ml respectively as in Fig.6.

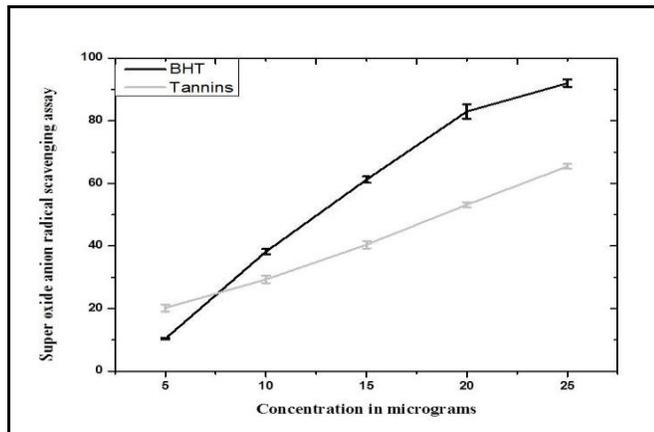


FIG.6: SUPER OXIDE ANION RADICAL SCAVENGING OF TANNINS (EC₅₀ value 19.0µg/ml) COMPARED TO BHT (EC₅₀ value 13 µg/ml)

Proapoptotic activity of tannins from *MM* on EAT cells: EAT cells treated with tannins when stained with Giemsa stain and observed under microscope confirmed the proapoptotic activity of the compound by displaying high nuclear condensation with clumping of nuclear chromatin. Blebbing of the nuclear and cytoplasmic membranes and few apoptotic bodies were seen as in Fig.7.

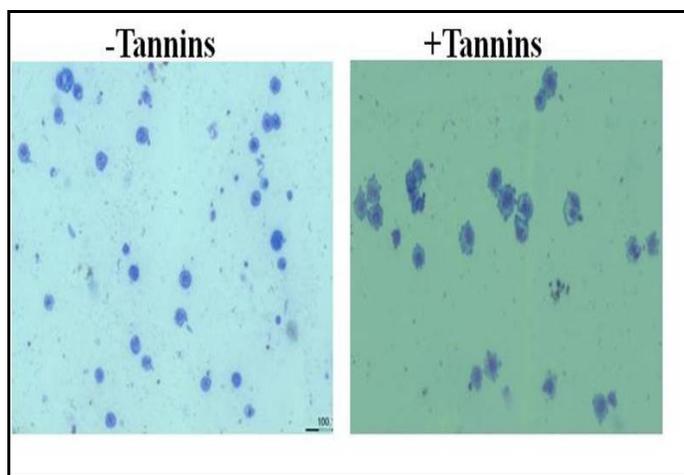


FIG.7: PROAPOPTIC ACTIVITY OF TANNINS FROM *MM* ON EAT CELLS SHOWING CYTOPLASMIC BLEBS AND APOPTIC BODIES (GIEMSA STAIN, X40).

The proapoptotic activity of the tannins on the EAT cells was further confirmed by Acridine orange-Ethidium bromide staining which demonstrated characteristic fluorescence under fluorescent microscope as in Fig. 8.

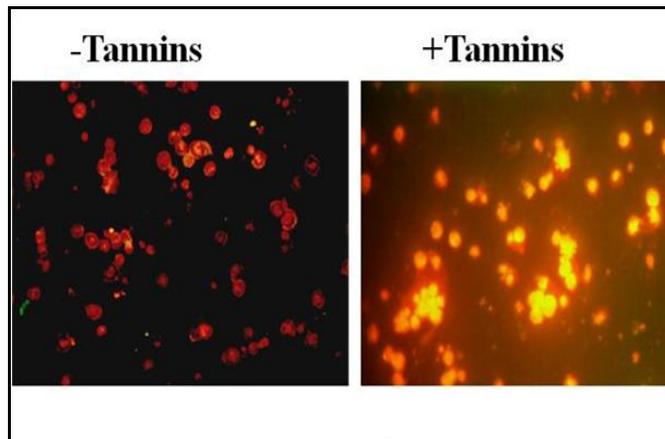


FIG.8: FLUORESCENT MICROSCOPY (ACRIDINE ORANGE: ETHEDIUM BROMIDE STAIN, X40) OF EAT CELLS TREATED WITH TANNINS FROM *MM* DISPLAYING CONDENSED NUCLEI AND FRAGMENTATION, SIGNIFYING PROAPOPTIC ACTIVITY

Effect of tannins from leaves of *MM* on DNA of EAT cells: Biochemically, apoptosis is characterized by fragmentation of chromosomal DNA. In the present investigation DNA isolated from EAT cells that were treated with tannins from *MM* when subjected to fragmentation assay demonstrated inter nucleosomal fragmentation of DNA leading to a DNA ladder formation. This confirms the proapoptotic role of the tannin on EAT cells as in Fig. 9.

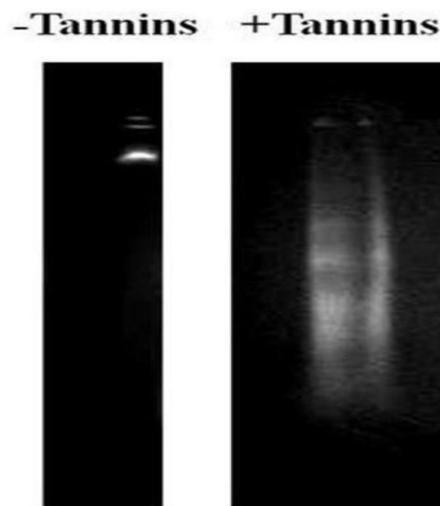


FIG.9: AGAROSE GEL ELECTROPHORESIS DISPLAYING DNA FRAGMENTATION IN EAT CELLS TREATED WITH TANNINS

DISCUSSIONS: Some of the molecular pathways that underlying the broad biological processes involved in initiation and proliferation of tumor are complex and multitude in nature. Oxidative stress,

angiogenesis, and the evasion of apoptosis are important biological mechanisms by which tumorigenesis and tumor progression occur. The primary focus of radiation therapy and chemotherapy is to produce irreversible DNA damage in tumor cells that will prevent their replication. While, other course of action is to alter cellular homeostasis, modify signal transduction pathways, redox state, arrest angiogenesis and promote disposition to apoptosis.

Antioxidants have proved to protect against oncogenic transformation by radiation and free radicals in experimental system²³. Supporting the beneficial effects of antioxidants in the present study is the high multiple antioxidant activity of the tannins. Especially the high free radical scavenging activity of the tannins from *MM* appear to protect the cells by neutralizing or trapping of reactive oxygen species and other free radicals. Free radicals react with lipid, cause peroxidation and results in release of products such as malondialdehyde (MDA), hydrogen peroxide (H₂O₂) and hydroxyl radicals (OH).

These reactive oxygen species (ROS) including O₂⁻, H₂O₂ and OH radicals play an important role in carcinogenesis²⁴. The moderate anti-hydroxyl and peroxide radical scavenging activities indicate that tannins from leaves of *MM* may involve in intonation of array of free radicals and subsequently cellular damages in tumorigenesis. High DNA abduction followed with pro-apoptotic and antiangiogenic activities ascertain competence of tannins from *MM* both as anti-initiating and antiproliferating tumor agent.

CONCLUSIONS: The use of leaves of *MM* which are primarily rich in a wide group of natural molecules such as polyphenols and tannins represent a promising class as anticancer drugs, due to their multipotent activity on cancer cells, with limited toxic effect on normal cells.

In the present investigation tannins isolated from *MM* has manifest their manifold activities like antiangiogenic, antioxidant and proapoptotic activity *in-vitro*, which can influence in therapeutic efficacy. Isolation of potent bioactive tannins would thus benefit the development of new generation of drugs. However, *in-vivo* evaluation

for its bioavailability and amelioration in tissues need to be defined

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