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PHYTOCHEMICAL SCREENING AND PHARMACOLOGICAL EVALUATION OF ANTICANCER ACTIVITY OF METHANOLIC EXTRACT OF MAYTENUS EMARGINATA (WILLD) IN RATS

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Abstract:
Cancer is one of the most serious health problems that affect the duration and quality of the individual's life. Enormous efforts are invested to cope with this problem, but unfortunately limited success has ever been achieved with most of the therapeutic strategies. These efforts are usually complicated with the need for well experienced surgeons, lack of specificity and high cost, as well as being usually accompanied with a wide range of side effects.
As the conventional therapeutic strategies fail to fulfill the major requirements for a successful cancer therapy, the use of naturally developed anticancer agents has evolved as an alternative safe, low-cost and convenient one. Therefore, the use of plant extracts with potential anticancer therapeutic effects might be particularly significant, especially in Palestine, which is rich in thousands of plant species known for their medical uses. Moreover, the lack of expertise, the scares economical resources and the complicated political situation in Palestine don't allow the application of sophisticated surgical, chemo- and radio-therapies to cure cancer.
Therefore, the current study, investigates the effect of crude methanolic extracts from Maytenus emarginata, Fig on cell lines derived from different human tissue origins (Hep3b: Hepatocellular carcinoma; Hela: cervical epithelial cancer; and A549: human lung adrenal cancer).
The results showed a concentration-dependent reduction in the final number of cancer cells in consequence to treatment with the aforementioned methanolic extracts. Two kinds of anticancer effects were evaluated and found tocontribute to this reduction: the antiproliferation effect (decreased number of metabolically active cells) and cytotoxicity (decreased number of live cells).
This extracts possess both of the effects with various degrees. Maytenus emarginata possess the strongest and most profound effects on the three cell lines, mainly by induction of cell death.
Further studies are needed to assess the active ingredients of Maytenus emarginata, involved in the antiproliferative or cytotoxic effects of these plants. These studies must involve the establishment of in vivo animal models and the application of more efficient extraction and fractionation techniques.

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INTRODUCTION:

The Indian subcontinent is a vast repository of medicinal plants that are used in traditional medical treatments [1]]. Many westerners have long regarded the Indian systems of medicine as a rich source of knowledge. In India, around 20,000 medicinal plants have been recorded however traditional communities are using only 7,000 - 7,500 plants for curing different diseases [2]. Even today, majority of the medicines are prepared from the plant and animal products, minerals and metals etc. Major pharmaceutical industries depend on the plant products for the preparation of Ayurvedic medicines.

Plants, especially used in Ayurveda can provide biologically active molecules and lead structures for the development of modified derivatives with enhanced activity and /or reduced toxicity. The small fraction of flowering plants that have so far been investigated have yielded about 120 therapeutic agents of known structure from about 90 species of plants. Some of the useful plant drugs include vinblastine, vincristine, taxol, podophyllotoxin, camptothecin, digitoxigenin, gitoxigenin, digoxigenin, tubocurarine, morphine, codeine, aspirin, atropine, pilocarpine, capscicine, allicin, curcumin, artemesinin and ephedrine [3]. In some cases, the crude extract of medicinal plants may be used as medicaments. On the other hand, the isolation and identification of the active principles and elucidation of the mechanism of action of a drug is of paramount importance. Hence, work in both mixture of traditional medicine and single active compounds are very important.

Pharmacognosy:

The word "pharmacognosy" was coined in the early 19th century to designate the discipline related to the study of medicinal plants [4]. The science of pharmacognosy became aligned with botany and plant chemistry, and until the early 20th century, dealt mostly with physical description and identification of whole and powdered plant drugs including their history, commerce, collection, preparation, and storage. Advances in organic chemistry added a new dimension to the description and quality control of these drugs, and the discipline has since expanded to include discovery of novel chemical therapeutic agents from the natural world [5]. Pharmacognosy studies help in identification and authentication of the plant material.

The process of standardization can be achieved by stepwise pharmacognostic studies [6]. The standardization of a crude drug is integral part of establishing its correct identity. Before any crude drug is included in herbal pharmacopoeia, pharmacognostic as well as other standard parameters must be established [7]. Therapeutic efficacies of medicinal plants depend upon the quality andquantity of chemical constituents. It has been established that chemical constituents of a plant species vary with regard to climate and seasons [8]. A number of different bases are used for morphological studies and a natural variation in these characteristics play an important role for preliminary evaluation of crude drugs. The basis of analysis by evaluation of microscopic characters is that there are always sufficient differences in the same type or different types of plants as for as the cell characteristics are concerned. Standardization profiles of herbal drugs are not available for most drugs [9].The therapeutic activity of herbs is because of various constituents present in them. Therapeutic efficacy of medicinal plants depends upon the quality and quantity of chemical constituents which may vary depending on various factors, one amongst is the geographical localities which show quantitative variation in their chemical constituents. In some plants toxic constituents are also present therefore it is essential to evaluate their quality, safety and efficacy. Correct identification and quality assurance of the starting material is therefore an essential prerequisite to ensurere producible quality of herbal medicine, which contributes to its safety and efficacy [10&11]. In most of the cases of herbal medicine, misuse starts with wrong identification. Many of the traditional systems have records where one common vernacular name is given to two or more entirely different species [12].

Macro and microscopic examination: For identification of right variety and search of adulterants.

Foreign organic matter: Remove matter other than source plant to get the drug in pure form.

Ash values: It is criteria to judge the identity and purity of crude drug - Total ash, sulfated ash, water soluble ash, acid insoluble ash, etc.

Moisture content: Checking moisture content helps reduce errors in the estimation of the actual weight of drug material. Low moisture content suggests better stability against degradation of product.

Extractive values: These are indicative weights of the extractable chemical constituents of crude drug under in different solvents.

Crude fiber: This helps to determine the woody material component and it is a criterion for judging purity.

Qualitative chemical evaluation: It helps in identification and characterization of crude drug with respect to phytochemicals constituent. It employs

different analytical techniques to detect and isolate the active constituents. Phytochemical screening techniques involve botanical identification, extraction with suitable solvents, purification and characterization of the active constituents of pharmaceutical importance.

Chromatographic examination: It involves identification of specific chemical constituents of crude drugs responsible for a specific activity and can be used as markers.

Quantitative chemical evaluation: To estimate the exact amount ofphytoconstituents present in the crude drugs.

Toxicological studies: Pesticide residue, potentially toxic elements, and microbial count which may reduce the efficacy of the final product.

Cancer is one of the most serious health problems worldwide, affecting individuals from different sexes, ages, and races. It is a group of diseases, characterized by uncontrolled cellular growth with frequent cancer cells invasion to different body parts and spreading to other organs, a process referred to as Metastasis. Metastasis is the major cause of cancer related mortality [13]. In 2005, cancer was the second leading cause of death among both men and women and accounted for 13% of the total 58 million deaths worldwide [13]. In 2006, about 10.9 million new cancer cases are expected to be diagnosed worldwide and more than 7.8 million cancer patients may die[13].According to the latest report of cancer registry unit in Gaza strip, 5500 cases have been reported over the period from January, 1995 to December, 2003[14]. In addition, 1026 cancer patients died in 2004 in the Palestinian territories with a mortality rate of 28.2 per 100.000 [14].

Cancer is also a problem of economical dimensions with a very high level of expenses associated to it. For example the National Institute of Health, USA estimates that an overall of $209.9 billion were invested worldwide in 2005, for the sake of cancer research and management [15]. Cancer is a heterogeneous illness which can originate from many different organs of the human body. However, the most frequent cancer types in the world are lung, prostate, stomach, colorectal, and esophagus in men; and breast, lung, stomach, colorectal and cervical in women.

Cancer (medical term: malignant neoplasm) is a class of diseases in which

- Group of cells display uncontrolled growth (division beyond the normal limits).
- Invasion (intrusion on and destruction of adjacent tissues).
- Sometimes metastasis (spread to other locations in the body via lymph or blood).

Three malignant properties of cancers differentiate them from benign tumors, which are self limited, and do not invade or metastasize.

1. Uncontrolled growth (division beyond the normal limit).
2. Invasion (intrusion on and destruction of adjacent tissues).
3. Metastasis (spread to other location in the body).

Nearly all cancers are caused by abnormalities in the genetic material of the transformed cells. These abnormalities may be due to the effects of carcinogens, such as tobacco smoke, radiation, chemicals, or infectious agents. Other cancer-promoting genetic abnormalities may be randomly acquired through errors in DNA replication, or are inherited.

Classification [16]:

Carcinoma: Malignant tumors derived from epithelial cells. Represents the most common cancers, including breast, prostate, lung and colon cancer.

Sarcoma: Malignant tumors derived from connective tissue, or mesenchymal cells.

Lymphoma and **leukemia:** Malignancies derived from hematopoietic (blood-forming) cells.

Germ cell tumor: Tumors derived from totipotent cells. In adults, found in the testicle and ovary.

Blastic tumor or blastoma: A tumor (usually malignant) which resembles an immature or embryonic tissue.

Molecular Biology of Cancer:

The following classes of genes have now been implicated in the development of cancer[17]

MATERIALS AND METHODS:

The designing of methodology involves a series of steps taken in a systematic way in order to achieve the set goal (s) under the prescribed guidelines and recommendations. It includes in it all the steps from field trip to the observation including selection and collection of the medicinal plant, selection of dose value, standardization of protocol, usage of instruments, preparation of reagents, selection of specific solvents for extraction, formation of protocols and final execution of the standardized protocol. All this requires good build of mind and a good and soft technical hand to handle the materials and procedure in a true scientific manner.

Drugs and Chemicals:

Drugs and Chemicals used in this study were of analytical grade and of highest purity procured from standard commercial sources in India.

Table1: Drugs and Chemicals

S.No	*Materials*	*Company Name*
1.	5-dephenyltetrazolium bromide	Sigma
2.	Dimethyl sulfoxide	Sigma Aldrich
3.	Trypsin	Sigma Aldrich
4.	Phosphate Buffer Saline	Fischer Bioreagent

Experimental animals:
Healthy adult albino wistar rats weighing 200-250grams of either sex were selected for the study. Animals were housed in appropriate cages in uniform hygienic conditions and fed with standard pellet diet (Amrul Laboratory Animal Diet) and water ad libitum. They were fasted overnight before the day of experiment. Animals were housed within the departmental animal house and the room temperature was maintained at 27° C. Animal studies had approval of IAEC.

Plant Material Collection:
Cell culture:
The human lung adreno carcinoma epithelial cell line A549 and human cervical carcinoma cell line HeLa were cultured in RPMI1640 supplemented with 10 % FBS and incubated in humidified atmosphere of 5 % CO_2 and 37 °C. The culture medium was changed every two days.

Cell viability assay:
The MTT assay, based on the conversion of the yellow tetrazolium salt-MTT, to purple-formazan crystals by metabolically active cells, provides a quantitative determination of viable cells (Mosmann, 1983). Cells were plated on to 96 well plates at a cell density of 2×105 mL^{-1} per well in 100 µL of (Rosewell park memorialinstitute 1640) RPMI 1640 and allowed to grow in a CO_2 incubator for 24 h (37 °C, 5% CO2). After 24 h, ME of *M.emarginata* and its fractions (0.1, 1, 10 and 50µg/ml) dissolved in DMSO was added to each well and incubated for 48 h. The control groups received the same amount of DMSO. Doxorubicin (0.01, 0.1, 0.5 and 1µg/ml) was used as positive control. The cells were incubated for 24-48 h (37 °C, 5 % CO2). Then, 100 µL MTT ([3-(4, 5-dimethylthiazol-yl)-2, 5-diphenyltetrazolium bromide]) solution (0.5 mg/mL in Dulbecco's modified eagle's medium) was added to each well and incubated for 3 h. Growth of tumoral cells was quantified by ability of living cells to reduce the yellow dye MTT to a blue formazan product. The formazan product of MTT reduction was dissolved in DMSO. The medium was removed and 100 µL DMSO was added to each well to dissolve the MTT metabolic product. Then the plate was shaken at 150 rpm for 5 min and the optical density was measured at 570 nm.

Percentage inhibitions [100 - (absorbance of test wells/absorbance of control wells) x 100] were calculated and plotted against the concentrations used to calculate the IC50 values.

ACUTE TOXICITY:
Experiment design:
The single dose toxicity test was conducted for 14 days according to Ryu et al. (2004). Wistar albino rats weighing between 180 and 250 g of either sex were used for acute toxicity study. The animals were divided into 4 groups of 6 animals. Fresh drug solutions were prepared in 5 % Tween 80 in water at the time of administration.

Grouping for acute toxicity study:
- Group I (Control): Animals received only 5 % Tween 80 in water on the 1st day of the study.
- Group II (Standard): Animals received standard (200 mg/kg, p.o.) on the 1st day of the study.
- Group III (400): Animals received (400 mg/kg, p.o.) on the 1st day of the study.
- Group IV (800): Animals received (800 mg/kg, p.o.) on the 1st day of the study.

Feed and water consumption and body weight measurement:
The amount of feed and water consumed was measured daily from the quantity of feed and water supplied and the amount remaining after 24 h for 14 days. Individual animal body weight was recorded daily till the end of the experiment.

Organ weight:
The various organs under study were excised from the animal and weighed. The weights of the organs such as liver, heart, lungs, thymus glands, spleen, adrenal glands, kidneys, testes, uterus and ovaries were recorded and studied for any abnormal gain or loss of weight. This gives a preliminary confirmation regarding the adverse effects (if any) of the drug under test. The weights of the organs expressed as relative weights as g/100 g b. w., were calculated by following formula:

$$\text{Relative organ weight} = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of rats on sacrifice day (g)}} \times 100$$

Hematological analyses:
On the 15th day, the animals were fasted for 12 h and then under mild ether anesthesia, animals were sacrificed and blood samples were collected. Blood was collected immediately into tubes containing EDTA for analysis of hematological parameters viz. hemoglobin, total red blood cells (R.B.C.), packed cell volume, mean cell volume (M.C.V.), mean cell hemoglobin (M.C.H.), mean cell hemoglobin concentration (M.C.H.C.), total white blood cells (W.B.C.), neutrophils, lymphocytes, eosinophils, monocytes, basophiles, total platelet count. The haematological analysis was doneusing hematology analyzer Sysmex XS800i (Sysmex corporation, USA).

Statistical analysis:
The data obtained from animal experiments are expressed as mean ± SEM (standard error of mean). For statistical analysis data were subjected to analysis of variance (ANOVA) followed by Student's t-test. Values are considered statistically significant at $F < 0.05$ for ANOVA and $P < 0.05$ for t-test.

RESULTS:

Phytochemical screening of M.emarginata:
The diagnostic characters of *M.emarginata* of fruit are the presence of quadrangular stem with winged corners and the internodes on four sides are invaded or depressed deeply in the middle and the corners are exerted with sharp reddish brown to black colored margins, 3-4 cm long (Fig. 2).

Microscopic characteristics:
Diagrammatic TS of the stem is four angled; on maturation each goes deep inside forming sharp pointed like projection and shows single layer epidermis followed by hypodermis; narrow cortex and centrally located large pith occupying almost 2/3rd region of the section, surrounded by numerous, small, discontinuous band of vascular bundles. Detailed section shows rectangular - pentagonal, 1-2 layered epidermis covered by thin cuticle, followed by 3-4 layered, circular-polygonal, chlorenchymatous hypodermis deposited more near the angle; cortex very narrow, cortical parenchymatous, 5-7 layered; pith very large, parenchymatous similar to that of region surrounded by discontinuous band of numerous, small, conjoint, collateral vascular bundles, each shielded with sclerenchymatous sheath, stele near the angle formed into strip, capped with collenchymatous band; few starch grains and rosette crystals and abundant large cells of mucilage, clusters and bundles of acicular crystals of calcium oxalate scattered throughout the section.

Powder characteristic:
The fine powder is green in color with faint odor. The diagnostic features of powder are plenty of cluster, rosette and acicular crystals of calcium oxalate scattered as such throughout or embedded in parenchymatous cells. Simple and compound starch grains 2-celled, scattered or embedded in parenchyma. Fragments of epidermis in surface view embedded with anisocytic stomata. The fibers are isolated or in groups, thin walled, occasionally exhibiting dentate margin, vessels with annular, reticulate and boarded pitted thickening. Cells of the medullary rays with pitted thickening.

Physicochemical analysis:
The results of physicochemical analysis of crude powder of *M.emarginata* are shown in Table 2. The average values of various parameters are expressed as percentage of air-dried material. Loss on drying was 9.5 %. Total ash was 19.41 %, acid insoluble ash was 17.0 % and water soluble ash was 14.16 %. The extractive value of crude powder was maximum in

water (19.18 %), followed by methanol (7.81 %) and minimum was in petroleum ether (1.11 %), pH of ME was 4.25

Solubility test:
The ME of *M.emarginata* fruiuts was evaluated for its solubility in 11 solvents with varied polarities. The extract was highly soluble in dimethylformamide, distilled water and methanol but less soluble in ethyl acetate, 1-4 dioxan and petroleum ether (Table 3). Determination of solubility of ME of *M.emarginata* fruits in different solvents.

Table 2: Physicochemical characteristics

Sr. No.	Physicochemical parameters	Average value % W/W
1.	Loss on drying	09.50%
2.	Total ash	19.41 %
3.	Acid insoluble ash	17.00 %
4.	Water soluble ash	14.16 %
5.	Petroleum ether soluble extractive	01.11 %
6.	Ethyl acetate soluble extractive	02.08 %
7.	Acetone soluble extractive	01.94 %
8.	Methanol soluble extractive	07.81 %
9.	Water soluble extractive	19.18 %
10.	pH (ME)	04.23

Phytochemical analysis:
Phytochemical analysis revealed the presence of secondary metabolites like alkaloids, flavonoids, cardiac glycosides and triterpenes (Table 4). However, the ME was rich in alkaloids (Wagner test) while crude powder was rich in cardiac glycosides.

Table-3 Phytochemical analysis:

No.	Phytochemical	Test	Crude Powder	Methanol extract
-1.	Alkaloids	Dragendroff test Mayer test Wagner test	+ + +	+ - +++
2.	Flavonoids	Shinoda test Alkaline reagent	- +	- -
3.	Cardiac glycosides	Keller-kilianni test	+++	++
4.	Tanins	HCl test FeCl3 test	- -	- -
5.	Saponins	Frothing test	-	+
6.	Steroids	Liebermann-Burchard reaction	+	+
7.	Triterpenes	H2SO4 test	+	+

Anticancer activity:
HeLa (Human cervical carcinoma) cell line:
The anticancer activity of ME of *M.emarginata* Fruits and its fractions against HeLa cell line was evaluated by MTT assay. Treatment with ME at concentration of 0.1, 1, 10 and 50 µg/ml for 24 h resulted in a concentration-dependent reduction in cell viability for HeLa cells. Estimated IC50 value for suppression of cell proliferation at 24 h was 12.40 and 15.34µg/ml (Table 7). Treatment with standard doxorubicin at concentration for 24 h resulted in a concentration-dependent reduction in cell viability for HeLa cells.

A549 (Human lung adenocarcinoma epithelial) cell line:
The anticancer activity of ME of *M.emarginata Fruits* and its fractions against A549 cell line was evaluated by MTT assay. Treatment with ME at concentration of 0.1, 1, 10 and 50 µg/ml for 24 h resulted in a concentration dependent reduction in cell viability for A549 cells. Estimated IC50 value for

suppression of cell proliferation at 24 h was 5.93 and 4.54µg/ml. (Table 5). Treatment with standard doxorubicin for 24 h resulted in a concentration dependent reduction in cell viability for A549 cells. Its IC50 value was 0.376 µg/ml (Table 5). ME showed good anticancer activity against A549 cell line than the HeLa cell line. Effect on ME on the proliferation of HeLa and A549 cells was studied in both cell lines, number of cells in treated plate (ME) reduced than in the control plate.

Table 4: The IC50 value of anticancer activity of ME of *M.emarginata Fruits*

Sr. No.	Sample	IC50 value (µg/ml)	
		HeLa	A549
1.	Control	20.5	9.54
2.	Doxorubicin	0.0701	0.376
3.	ME(400)	12.40	5.93
4.	ME(800)	15.34	4.54

DISCUSSION:

Pharmacognostic studies:

The main diagnostic microscopic features of Maytenus emerginata were showed the presence of three types of crystals such as rosettes, clusters and bundles of acicular. These three types of calcium oxalate crystals were found both in intact stem and powder form.

The physicochemical constants are important parameters for detecting adulteration or improper handling of drugs. The percentage of active chemical constituents in crude drugs is mentioned on air-dried basis; therefore, the loss on drying of plant materials should be determined. The moisture content of dry powder of stem was 9.5 % which is not very high, hence it would discourage bacteria, fungi or yeast growth.

The important microscopic features of the plant were transverse section of stem showing single layered epidermis followed by hypodermis and narrow cortex; midrib showing both upper and lower continuous epidermis; arc shaped vascular bundles; all three types of calcium oxalate crystals scattered throughout.

Maytenus emerginata. is used for the treatment of various diseases therefore it is important to standardize it for use as a drug. The pharmacognostic constants for the stem of this plant, the diagnostic microscopic features and the numerical standards reported in this work could be useful for the compilation of a suitable monograph for its proper identification.

Physicochemical analysis:

The residue remaining after incineration of plant material is the ash content or ash value, which simply represents inorganic salts, naturally occurring in crude drug or adhering to it or deliberately added to it, as a form of adulteration. The ash value was determined by three different methods viz. total ash, acid-insoluble ash and water soluble ash.

The total ash method is employed to measure the total amount of material remaining after ignition. This includes both 'physiological ash' which is derived from the plant tissue itself, and 'non-physiological ash', which is the residue of the extraneous matter adhering to the plant surface. Acid-insoluble ash is a part of total ash and measures the amount of silica present, especially as sand and siliceous earth.

Water soluble ash is the water soluble portion of the total ash (Kokate et al., 2006; Dave et al., 2010**)**. These ash values are important quantitative standards. All these three parameters were determined in *Maytenus emerginata*.

Phytochemical analysis shows the presence of many medicinally important secondary metabolite types of phytoconstituents like alkaloids, cardiac glycosides, saponins, triterpenes, which indicates that the plant possesses high profile values and can be used to treat various kinds of diseases. The qualitative phytochemical investigation gave valuable information about the different phytoconstituents present in the extracts, which helps the future investigators regarding the selection of the particular extract for further investigation of isolating the active principle.

Alkaloid compounds are considered to be the most important anticancer of plant materials. Anticancer activity of alkaloid compounds is based on their ability to reduce the cytotoxicity to the cell. Alkaloids are widely present in plant kingdom particularly in ethanobotanically important plants. Alkaloids have

been involved in bioactivities of plants including anticancer, antimalarial and antiviral as well. Alkaloid may contribute directly to anti-cancer action. It is known that alkaloid compounds have inhibitory effects on mutagenesis and carcinogenesis in humans when up to 1 g daily is consumed from a diet rich in fruits and vegetables.

CONCLUSION:

Maytenus Emarginata belongs to the family Celastraceae, an edible plant found in India. It is commonly known as "Tho My Soft Tree". The fruits of *Maytenus Emarginata* are reported to have great medicinal value. Considering above, in the present work, anticancer and toxicity study was evaluated.

In physicochemical analysis, the highest extractive value was obtained from water and methanol extract. The extract was maximum soluble in polar solvents like DMF, methanol and water; and was acidic in nature.

In qualitative phytochemical analysis, cardiac glycosides and alkaloids were present in higher amount, while tannins were totally absent. The quantitative phytochemical investigation gave valuable information about the different phytoconstituents present in the powder extract, which helps the future investigators regarding the selection of the particular extract for further research in isolation of new active compounds. The total phenol content was higher than flavonoid content. Such Pharmacognostic study serves important criteria in standardization of the *Maytenus Emarginata* fruit, ensuring quality formulations.

In anticancer studies, ME showed potent proliferation inhibitory activity against human lung adenocarcinoma epithelial cell line (A549) and human cervical carcinoma cell line (HeLa). This is the first report on the anticancer properties of *Maytenus Emarginata*. The ME showed good anticancer activity against A549 cell line than the HeLa cell line.

In acute toxicity study, there is no mortality and observable acute toxic effect during the entire period in male and female rats dosed up to 800 mg/kg b.w. orally. Detailed experimental analysis on sub acute and chronic toxicity is essential for further support of this drug.

These studies have shown that the ME of *Maytenus Emarginata* and its fractions contain some active ingredients with the potential of being good anticancer agents. Further work should be carried out on the characterization of specific antioxidant and anticancer components of *Maytenus Emarginata* and evaluation of their therapeutic significance in prevention of diseases induced by oxidative stress.

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