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RESEARCH ARTICLE

In Vitro Anti-cancer Activities of Chinese Chaste Tree and Indian Turnsole against Pc3 and Hela Cell Lines

Kuldeep Jaiswal, Ragini Bundela, Sourabh Jain, Karunakar Shukla

Department of Pharmacology, College of Pharmacy, Dr. A. P. J. Abdul Kalam University, Indore, Madhya Pradesh, India

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ABSTRACT

Natural products discovered from medicinal plants have played an important role in the treatment of cancer. The present study points to the potential anticancer activity of chloroform and ethanol extract of *Vitex negundo* and *Heliotropium indicum*. Further studies to characterize the active principles and elucidate the mechanism of the action of ethanol and chloroform extract are in progress. Hence, these plant extracts may have clinical and therapeutic proposition in the most life threaten disease like cancer and further studies are required to investigate these plant samples as antineoplastic agents. Therefore, it is anticipated that plants can provide potential bioactive compounds for the development of new "leads" to combat cancer diseases. The chloroform, ethanol, and aqueous extract of *V. negundo* have shown potent anticancer activity on selected cancerous cell lines. To provide scientific validation to the traditional use of plants *in vitro* cytotoxic effect was evaluated on HeLa cell lines. More efforts are needed to explore potent anticancer plants from the mother earth and save humans around the world from cancer.

Keywords: Bioassay, he.la cell lines, *Heliotropium indicum, In vitro, Vitex negundo*

INTRODUCTION

Cell division in humans is mainly controlled by DNA of the cell. Main factors are responsible for the cause of cancer such as chemical carcinogens, viruses, chromosomal rearrangement or spontaneous transformation, and tumor suppressor genes. Cancer can be caused by any of the three ways improper diet, genetic factors, and environmental factors.[1,2] More than 35% of all cancers worldwide are caused by improper diet in the case of colon cancer; diet may account for more than 80% of the cases. Alcohol and cigarettes to their diet, the percentage cause of cancer may increase to 60%. Plants have been demonstrated clinical source for anticancer compounds. However, many of the plant products and their derivatives are approved for cancer control. Hence, the development of new drugs to play an important role in cancer control is greatly desired.[3] *Vitex negundo* Linn (Synonym: *Vitex incise* Linn, *Vitex incise* Lam *Var hetropylla*., family: *Verbanaceae*). A large and aromatic shrub or sometimes a small slender tree, up to 4.5 m in height, found throughout the greater part of India. Leaves possess anti-inflammatory, analgesic, and antihistamine properties. Roots are used for leprosy, dyspepsia, rheumatism, and piles. Bark is used as verminosis and opthalmopathy. Flowers are used in cholera. Fruit used as anthelmintic. The whole plant is used in inflammations, antiseptic, antipyretic, and diuretic.[4-10] Earlier studies have shown that the plant possess anti-inflammatory and antihistamine,[11] analgesic,[12] antioxidant,[13] antibacterial,[14] CNS depressant,[15] antifungal,[16] snake venom neutralization,[17] mosquito repellant activity,[18] insecticidal,[19] larvicidal efficacy,[20] antinociceptive,[21] antiandrogenic,[22] Hepatoprotective,[23] antifertility,[24] skin aging inhibitor,[25] and anti-dopaminergic[26] effects. Constituents previously isolated from the plant

***Corresponding Author:**
Sourabh Jain,
E-mail: drsourabh294@gmail.com

include eight lignans[27] (negundin A, negundin B, 6-hydroxy-4-(4-hydroxy -3 methoxy)-3- hydroxyl methyl -7 methoxy -3,4 di hydro 2 napathaldehyde, vitrofolal, (+) – iynoiresinol, (+) – iynoiresinol -3α-0-β-Dglucoside, (+)(-)(-) pinorecinol and (+) –diasyringaresinol, irridoid glycoside (2-p-hydroxy benzoyl mussaenosidic acid), flavonones (5,3' di hydroxyl -7,8,4' trimethoxy flavonone and (5,3' dihydroxy -6,7,4' trimethoxy flavonone), flavones (vitexicarpin), β – sitosterol, essential oils (α –pinene, linalool, terpinyl acetate, and beta caryophyllene), non-diterpene. (vitedoin B), pentacyclic triterpenoids (beutinilic acid and ursolic acid) and flavonoid glycoside (luteolin, agnuside, negundoside, and iso-orientin). *Heliotropium indicum* has been used widely for centuries on warts and to treat inflammations and tumors. *H. indicum* Linn (Family Boraginaceae) is a medicinal plant. It is known commonly as "Cock's comb". *H. indicum* Linn has various medicinal uses in the treatment of disease conditions such as abdominal pains, amenorrhea, dysmenorrhea, skin rashes, wounds, hypertension, ocular infections, convulsion, and dizziness. To provide scientific validation to the traditional use of plants, *in vitro* cytotoxic effect was evaluated on HeLa cell lines.[28]

MATERIALS AND METHODS

Plant Materials

Preparation of extracts of V. negundo and H. indicum

The powdered leaves (1 kg) were sequentially extracted using chloroform and ethanoland aqueous solution in Soxhlet apparatus. After about forty siphons of each solvent extraction step, the materials were concentrated by evaporation.

Preliminary phytochemical screening

Extracts of *V. negundo* and *H. indicum* were subjected to qualitative tests for the identification of various active constituents, namely, carbohydrate, glycoside, alkaloid, amino acids, flavanoids, fixed oil, tannins, gum and mucilage, and phytosterols. The phytoconstituents were identified by chemical tests, which showed the presence of various constituents in the different extracts.[29,30]

Brine shrimp lethality bioassay

The cytotoxic potential of extract of *V. nugendo* and *H. indicum* was determined by brine shrimp lethality bioassay. Briefly, eggs of brine shrimp Artemiasalina were hatched in a container filled with air bubbled artificial sea water which was prepare dusing 10 g of a commercial salt mixture (GEX Inc., Osaka, Japan) and 500 mL of distilled water. After 36–48 h, the phototropic shrimps were collected and used for bioassay. To the vials containing different concentrations of extracts in sea water (1, 10, 25, 50, and 100 μg/mL), 25 shrimps were added and the vials were incubated at 25°C and the surviving shrimps were counted after 24 h. The LC_{50} values of extracts >1000 μg/mL were considered inactive (non-toxic). Potassium dichromate was used as reference standard.

In Vitro Anticancer Activity

Cell lines and culture conditions

Human cervical cancer cell line (HeLa) and Human prostate cancer cell line (PC3) cell lines were procured from NCCS, Pune, India. Cells were grown in Minimum Essential Medium Eagle (Gibco, UK) supplemented with 10% heat inactivated fetal bovine serum (Gibco, UK), 29 μg/mL L-glutamine, and 40 μg/mL Gentamicin. Cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C.

Antiproliferative activity

The antiproliferative activity of plant extracts was measured using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) assay(Promega,USA). The assay detects the reduction of MTT by mitochondrial dehydrogenase to blue Formazan product, which reflects the normal function of mitochondria and cell viability. Exponentially growing cells were washed and seeded at 17,000 cells/well (in 200 μL of growth medium) in 96 well microplates (Nunc, Denmark). After 24 h incubation, a partial monolayer was formed then the media was removed and 200 μL of the medium containing the plant extract (initially dissolved in DMSO) were added and reincubated for 48 h. Then, 100 μL of the medium were aspirated

and 15 µL of the MTT solution were added to the remaining medium (100 µL) in each well. After 4 h contact with the MTT solution, blue crystals were formed. 100 µL of the stop solution were added and incubated further for 1h. Reduced MTT was assayed at 550 nm using a microplate reader (Das, Italy). Control groups received the same amount of DMSO (0.1%).Untreated cells were used as a negative control while, cells treated with vincristine sulfate were used as a positive control at the following concentrations 0.05, 0.1, 0.5, 1,5, 10, 25, 50, and 100 nM. IC_{50} values were calculated as the concentrations that show 50% inhibition of proliferation on any tested cell line. Stock solutions of the plant extract were dissolved in (DMSO) then diluted with the medium and sterilized using 0.2 µm membrane filters. The final dilution of extracts used for treating the cells contained not more than 0.1% DMSO. Data were reported as the average of three replicates. The antiproliferative effect of the tested extracts was determined by comparing the optical density of the treated cells against the optical density of the control (untreated cells).

DNA fragmentation assay

To determine the extracts induce apoptosis in SiHa cells, DNA fragmentation assay by agarose gel electrophoresis was performed. The cells (1×10^6) were seeded in 60 mm tissues culture dish treated with or without drug and incubated for 48 h. Cells were harvested by centrifugation and lysed in ice for 30 min by the addition of 20 µL lysis buffer contains 20 mM EDTA, 100 mM Tris (pH 8.0), and 0.8% (w/v) sodium lauryl sarcosine. The lysates was digested with RNase A (2 µL, 5 mg/mL) and proteinase K (20 µL, 10 mg/µL) at 37°C for 1 h and 2 h, respectively. Total lysates were loaded onto 1.5% agarose gel stained with 0.5 µg/mL ethidium bromide and separated at 50 mV. DNA fragments were visualized by the Gel Doc100 system (Bio-Rad; Hercules, CA).

Table 1: Successive extraction of chloroform, ethanol, and aqueous extracts of Vitex negundo and Helio tropium indicum

S. No	Extracts	Percentage yield of extracts of Vitex negundo w/w	Percentage yield of extracts of Helio tropium indicum w/w
1.	Chloroform	4.23	2.12
2.	Ethanol	5.35	8.34
3.	AqueouS	13.8	9.15

Statistical Analysis

All experiments were repeated at least 3 times. At least quadruplicate cultures were scored for an experimental point. All values were expressed as mean ± S.E.M. The Student's one tail t-test was applied for statistical treatment of the results; $P < 0.05$ was considered as the statistically significant value.

RESULTS AND DISCUSSION

The preliminary phytochemical screening of *V. nugendo* and *H. indicum* leaf extracts [Table 1] showed presence of steroids and sterols, triterpenoids, alkaloids, flavonoids, saponins, tannins and phenolic substances, gums and mucilages, carbohydrates, and proteins, respectively, in different extracts [Table 2]. The result of cytotoxic potential of ethanol extract of *V. nugendo* and *H. indicum* in terms of mortality of brine shrimps (%) is presented in Figure 1. The degree of lethality was directly proportional to the concentration of the extracts. The percentage mortality of shrimps [Table 3] was recorded higher in case of *V. nugendo* LC_{50} were 50 and 25 µg/mL

Table 2: Phytochemical screening of chloroform, ethanol, and aqueous extracts of Vitex negundo and Heliotropium indicum

Tests	Vitex negundo			Helio tropium indicum		
	CE	EE	AE	CE	EE	AE
Alkaloids	+	+	-	+	+	-
Carbohydrates	-	-	+	-	-	+
Glycosides	-	-	-	-	-	-
Gums and mucilages	-	-	+	-	-	+
Proteins and amino acids	-	-	-	-	+	-
Tannins and phenolic compounds	-	+	-	-	+	-
Steroids and sterols	+	+	-	+	-	-
Triterpenoids	+	-	-	+	-	
Saponins	-	-	+	-	+	+
Flavonoids	-	-	+	-	+	+

(+) indicates presence and (-) indicates absence of phytochemicals, CE: Chloroform extract, EE; Ethanol extract, AE: Aqueous extract

Table 3: Brine shrimp lethality bioassay of Vitex negundo and Helio tropium indicum

Conc.(µg/mL)	Log C	% Mortality (Vitex negundo)			% Mortality (Helio tropium indicum)		
		Chloroform extract	Ethanol extract	Aqueousextract	Chloroform extract	Ethanol extract	Aqueous extract
200	2.301	100	90	100	90	100	100
100	2.000	80	80	100	80	80	100
50	1.699	50	70	100	70	70	100
25	1.398	45	50	90	50	50	90
10	1.010	40	40	80	40	50	80
5	0.750	30	30	70	30	40	70
3	0.450	20	20	70	20	40	60
1	0.150	10	10	60	10	30	50

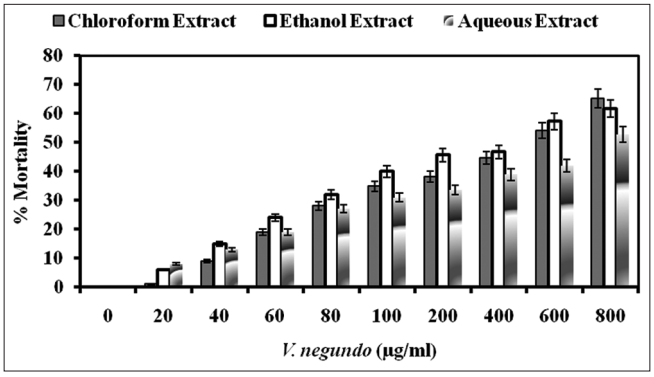

Figure 1: Brine shrimp lethality bioassay of *Vitex negundo*

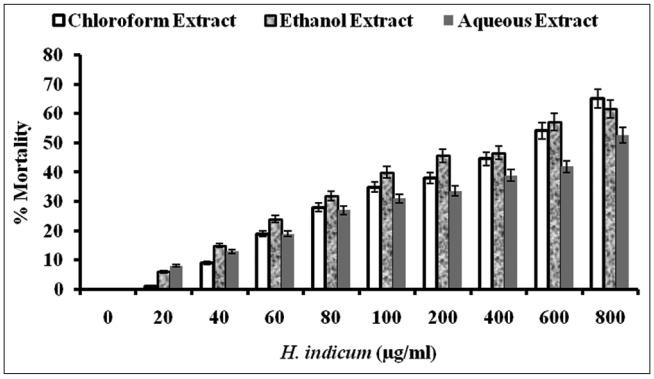

Figure 2: Brine shrimp lethality bioassay of *Helio tropium indicum*

of chloroform and ethanol extract, respectively [Figure 1] than that of *H. indicum* LC_{50} were 25 and 25 µg/mL of chloroform and ethanol extract, respectively [Figure 2]. Extract of *H. indicum* showed more lethality when compared with the reference control, that is, potassium dichromate (LC_{50} 32.77 µg/mL). Highest mortality (100%) was observed at concentration 100 µg/mL of both the extracts. The aqueous extracts of both the plats did not show any mortality in brine shrimp assay.

PC-3 and HeLa were exposed to chloroform and ethanol extract of *V. nugendo* and *H. indicum* for 24 h and cytotoxicity was determined with the MTT assay. The percentage cancer cell inhibition profiles were found to be concentration dependent [Figures 3-6]. The maximum concentration used in the study was 500 mg/mL. HeLa cell lines, when subjected to different concentrations of plant extracts displayed weak inhibition of 31.25%. It was observed from Figures 3-6 that a gradual increase in percentage inhibition was observed in all the cases. The extracts of *V. nugendo* exhibited significant cytotoxic activity against

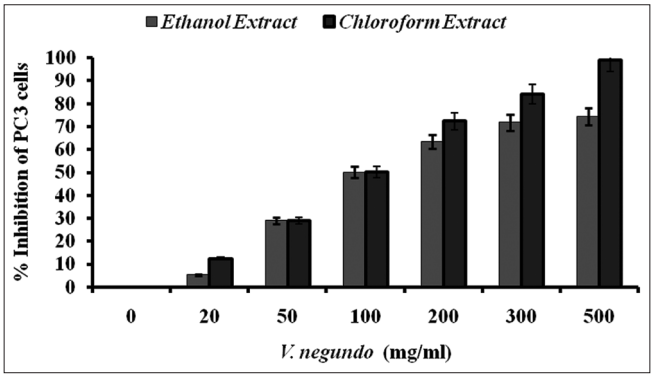

Figure 3: Antiproliferative activity of extracts of *Vitex negundo* in PC3 cellline

PC3 cell line with IC_{50} values of 70.23 mg/mL and79.02 mg/mL for chloroform and ethanol fraction, respectively [Figure 3], where good cytotoxicity was shown against HeLa cells with IC_{50} values of 48.13 mg/mL and 59.87 mg/mL for chloroform and ethanol fraction, respectively [Figure 5]. Similarly, the extracts of *H. indicum* exhibited IC_{50} of 67.23 mg/mL and 72.23 mg/mL for chloroform and ethanol fraction, respectively [Figure 4], where good cytotoxicity was shown

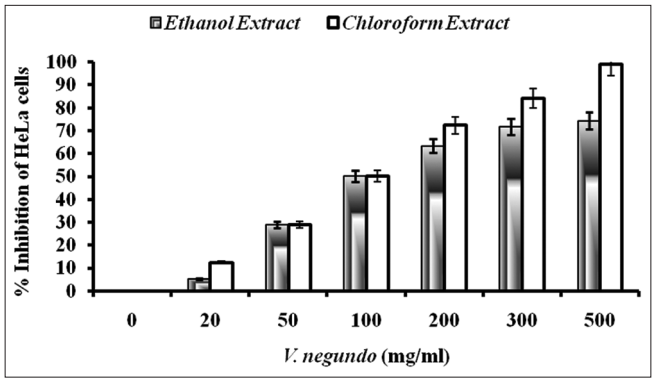

Figure 4: Antiproliferative activity of extracts of *Vitex negundo* in HeLa cellline

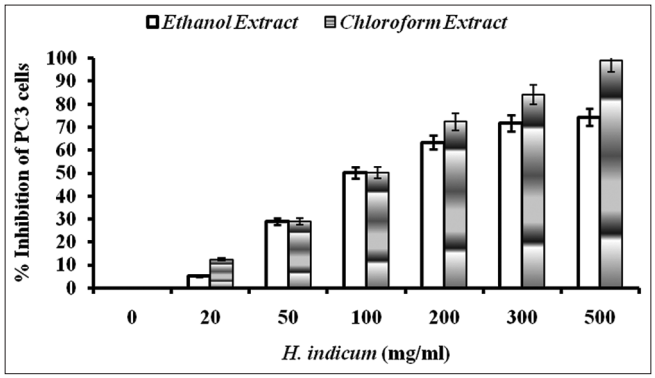

Figure 5: Antiproliferative activity of extracts of *Helio tropium indicum* in PC3 cellline

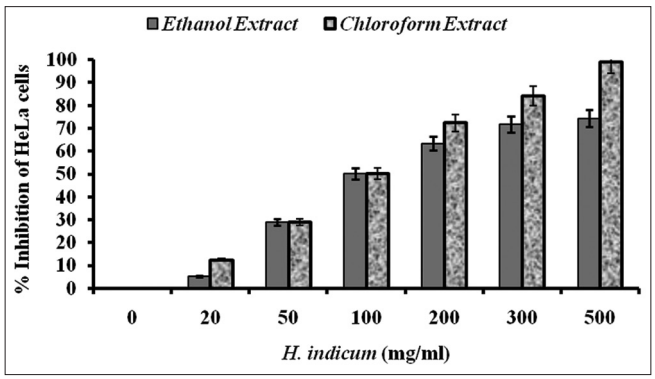

Figure 6: Antiproliferative activity of extracts of *Helio tropium indicum* in HeLa cellline

against HeLa cell line with IC_{50} values of 58.43 mg/mL ad 64.23 mg/mL for chloroform and ethanol fraction, respectively [Figure 6]. Whereas, aqueous extracts of both the plants were found to be non-toxic in both the cell lines. Induction of apoptosis on HeLa and PC3 cells by *V. nugendo* and *H. indicum* extracts was validated by DNA fragmentation analysis using gel electrophoresis technique. The DNA bands obtained from both extract-treated HeLa and PC3 produced ladder

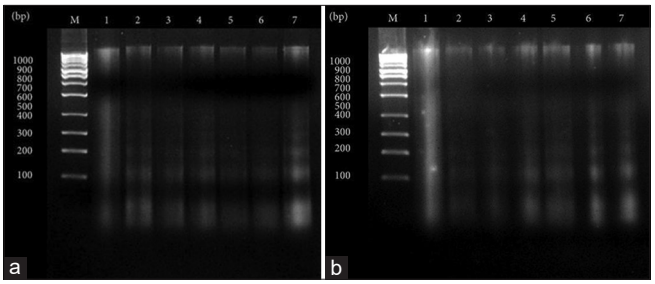

Figure 7: DNA band patterns of (a) HeLa and (b) PC3 cells treated with various concentrations of *Helio tropium indicum* and *Vitex negundo*. Lane1: negative control; Lanes 2 to 3 were bands of cancer cells treated with 100 and 200 mg/mL extract of *Vitex negund.* Lanes 4 to 7 with 100 and 200 mg/mL extract of *Helio tropium indicum*

pattern as observed from Lane 2 to 7 [Figure 7]. A ladder formation was used to indicate that the DNA has undergone fragmentation, and each fragment corresponded to a band in the ladder.

DISCUSSION

Ever since the existence of human being, plants have been exploited for several purposes including medicinal purposes. Plants are the primary source of biologically active phytochemicals present in conventional medicaments. Medicinal systems, namely, Ayurveda, Unani, and Sidda employ the use of these plants for the treatment of diseases. Ethnobotanical studies highlight the relationships between various cultures and the traditional use of plants. Several ethnic groups all over the world employ a number of plant species for treatment of various ailments ranging from mild infections to fatal infections. Often, these studies are of importance and provide essential information for development of scientific research to justify the therapeutic potential of plants. Brine shrimp lethality bioassay is an *in vivo* lethality assay that employs a simple zoologic organism as a convenient monitor for screening, discovering, and monitoring various bioactivities of natural compounds. This test is very useful in determining various biological activities such as cytotoxic, phototoxic, pesticidal, trypanocidal, enzyme inhibition, and ion regulation activities. The assay can also be extrapolated for cell-line toxicity and antitumor activity. The method is rapid as it utilizes only 24 h, inexpensive, and needs no special equipment. It is even simple in that

it does not require aseptic conditions to perform. The assay employs large number of organisms for validation and a relatively small amount of sample. This bioassay has been employed to determine cytotoxic activity of plant extracts.[31-34] In our study, the ethanol extract of *V. negundo* and *H. indicum* displayed cytotoxic activity as evidenced by the dose dependent mortality of brine shrimp larvae. Among extracts, higher cytotoxicity was observed in case of *H. indicum* extract than that of extract of *V. negundo*. High mortality of shrimps caused by extract of *H. indicum* could be described to the presence of high phenolic and flavonoid content. In another study[35] showed cytotoxic activity in terms of brine shrimp mortality of two compounds isolated from leaves of *V. negundo*. Crude ethanol extract and solvent fractions of bark of *V. negundo* were shown to exhibit marked cytotoxic effect in terms of mortality of brine shrimp larva. The reported results show that ethanolic extract of *H. indicum* has significant anticancer effect on prostate cancer (PC-3 cell line) compared to cervical cancer cell line (HeLa). The earlier work revealed that the ethanol extract of *H. indicum* leaves possess cytotoxic and anticancer properties. Apoptosis is generally considered an energy-dependent process requiring active participation of many proteins and other cellular macromolecules. It is due to the fact that most of the intense genotoxic stimuli damage the proteins (or genes which are making those proteins) and other cellular macromolecules which may be required for apoptosis. The damage to proteins would result in their denaturation. This denaturation would confine the damaged DNA to the nuclear area giving a sharper outline to the nuclear boundary in necrotic cells. This sharpness in outline in necrotic cells may also be due to larger sized DNA, which does not diffuse as it does in apoptotic cells where DNA is as small as 180 bases. Verification of the apoptotic activity was carried out based on the pattern of DNA bands produced from a gel electrophoresis. In apoptosis, cells are lysed gradually and systematically to produce membrane-bound apoptotic bodies, which was suggested to play a major role in suppressing inflammatory responses to other neighboring cells. Apoptotic bodies or cells which underwent apoptosis produce a specific pattern of DNA fragments with the multiples of 200 bp due to specific action of activated nucleases. These isolated fragments produced bands in a ladder pattern, in contrast with the smeared pattern produced from necrosis activity [Figure 7]. Results obtained in this study in a way supported the various claims made by researches on the anticancer properties of these plant.[10-15] Further studies are being carried out to identify the active principle of the extract. Thus, it can be concluded that the strong antiproliferative activity of extract on cancer cells suggests its possible development as an anticancer agent. The mode of action of the extract was by the induction of apoptotic activity on cancer cells.

CONCLUSION

Natural products discovered from medicinal plants have played an important role in the treatment of cancer. The present study points to the potential anticancer activity of chloroform and ethanol extract of *V. negundo* and *H. indicum*. Further studies to characterize the active principles and elucidate the mechanism of the action of ethanol and chloroform extract are in progress. Hence, these plant extracts may have clinical and therapeutic proposition in the most life threaten disease like cancer and further studies are required to investigate these plant samples as antineoplastic agents. Therefore, it is anticipated that plants can provide potential bioactive compounds for the development of new "leads" to combat cancer diseases.

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