Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Combinatorial Effect of *Macrotyloma uniflorum* (Lam.) Verdc. Seed Extract and Vorinostat (Suberoylanilide Hydroxamic Acid) in Human Leukemia Cells

Azra Anjum, Humaira Farooqi, M.Z. Abdin, Ashok Kumar Tiwari[1]

Department of Biotechnology, School of Chemical and Life Sciences, Jamia Hamdard, New Delhi, [1]Medicinal Chemistry and Pharmacology Division, CSIR-Indian Institute of Chemical Technology, Hyderabad, Telangana, India

Submitted: 12-Feb-2021 Revised: 21-May-2021 Accepted: 04-Jul-2021 Published: 15-Sep-2021

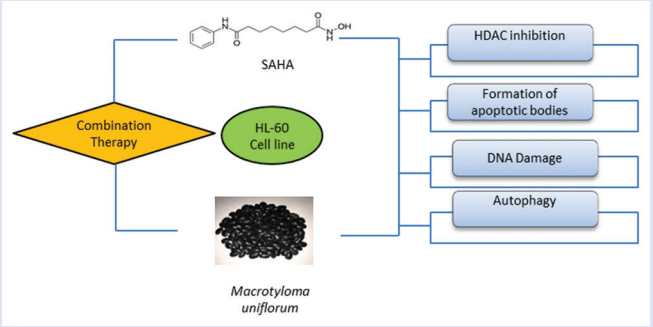
ABSTRACT

Background: Many histone deacetylase (HDAC) inhibitors have demonstrated preclinical efficacy as monotherapy or in combination with other anticancer drugs for both hematological and solid tumor malignancies. In clinical, however, the HDAC inhibitors have proven less effective as single agents. Thus, prompting the investigation of rational combination of HDAC inhibitors with other therapeutics. **Objectives:** This study aims to evaluate the potential of *Macrotyloma uniflorum* in facilitating the potency of histone deacetylase inhibitor, Vorinostat-suberoylanilide hydroxamic acid (SAHA). **Materials and Methods:** Powdered seeds of *M. uniflorum* were sequentially extracted with water, methanol, ethyl acetate, and hexane. The quantitative analysis of the different extracts was carried out. The cytotoxicity of *M. uniflorum* seed extracts was investigated on the leukemia cell line (HL-60). HeLa cells were used to study the histone deacetylase inhibitory activity of the extracts. Further, the effect of the combination of *M. uniflorum* seed extract and SAHA was studied. **Results:** The aqueous extract showed the highest amount of both phenolic (311.21 ± 2 mg gallic acid/g) and flavonoid content (89.96 ± 4.62 mg Quercetin/g extract). The combination showed more specificity toward class I HDAC isoforms among the different isoforms (class I, class IIa, class IIb, and class IV). Moreover, *M. uniflorum* alone showed no significant cytotoxicity on the HL-60 cell line. However, in combination with SAHA, the aqueous extract exhibited significant cytotoxic activity. Further, the results show that the extract of *M. uniflorum* has a significant effect on cell death and autophagy in leukemia. **Conclusion:** Mechanistic studies revealed that aqueous extract along with SAHA facilitated HDAC inhibitory activity, cell death, and autophagy.

Key words: Anticancer, bioactive constituents, combination therapy, histone deacetylase inhibition, suberanilide hydroxamic acid

SUMMARY

- The authors of this study evaluated the combinatorial effect of *Macrotyloma uniflorum* aqueous extract along with suberoylanilide hydroxamic acid (SAHA) on the leukemia cell line. It is documented that the natural bioactive compounds can act in synergy with anticancer drugs. Our results show that the combination induced significant inhibition and apoptosis of HL-60 cells. Cellular morphological alterations and cell death confirmed apoptosis caused by the combination. These findings are important because apoptotic machinery control is central in the development of cancer disease. Among the various extracts (aqueous, methanol, ethyl acetate, and hexane) aqueous extract showed the highest amount of both phenolic and flavonoid content. The combination showed more specificity towards class I histone deacetylase isoforms among the different isoforms (class I, class IIa, class IIb, and class IV). Moreover, *M. uniflorum* alone showed no significant cytotoxicity on the HL-60 cell line. However, in combination with SAHA, aqueous extract exhibited significant cytotoxic activity. Further, the results show that the extract of *M. uniflorum* has a significant effect on cell death and autophagy in leukemia.



Abbreviations used: SAHA: Suberanilide hydroxamic acid; HDAC: Histone deacetylases; HDACi: Histone deacetylase inhibitor; HAT: Histone acetyltransferases; TPC: Total phenol content; TFC: Total flavonoid content; PBS: Phosphate-buffered saline; DAPI: 4′, 6-diamidino-2-phnylindole.

Correspondence:

Dr. Humaira Farooqi,
Department of Biotechnology, School of Chemical and Life Sciences, Jamia Hamdard, New Delhi - 110 062, India.
E-mail: hfarooqi@jamiahamdard.ac.in

DOI: 10.4103/pm.pm_70_21

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Epigenetics has been shown to play a crucial role in the initiation and development of cancer.[1] DNA methylation and histone modification are normal epigenetic changes and dysregulation induced by the shift in amino acid sequence of these enzymes is closely associated with the emergence and progression of various types of cancers.[2] Histone acetylation is a fundamental process which regulates cellular activities like histone acetylation and histone deacetylation.[3] These two processes are controlled by two antagonistic families of enzymes, histone deacetylases (HDACs) and histone acetyltransferases respectively.

Cite this article as: Anjum A, Farooqi H, Abdin MZ, Tiwari AK. Combinatorial effect of *Macrotyloma uniflorum* (Lam.) Verdc. Seed extract and vorinostat (Suberoylanilide hydroxamic acid) in human leukemia cells. Phcog Mag 2021;17:S213-9.

It has been observed that the overexpression of HDACs can lead to tumorigenesis and therefore, histone deacetylase inhibitors (HDACi) have been approved as promising anticancer drugs.[4] HDACi belong to a category of drugs which modulate the chromatin structure through histone acetylation and can determine changes in gene transcription. They also effect biological processes which are directly associated with the suppression of cancer which includes the arrest of cell growth and differentiation.[5] Suberoylanilide hydroxamic acid (SAHA) was the first HDACi approved by the US Food and Drug Administration (2004) for the treatment of cutaneous T-cell lymphoma.[6]

Based on the potential therapeutic efficacy of HDACi various classes have been designed which have proven effective in various malignancies, either alone or in combination with other anticancer drugs.[7] Clinically, however, the efficacy of HDACi as single agents is not satisfactory, and hence, a need to investigate an appropriate combination of HDACi with other therapeutics.[8] In addition to the HDACi being produced as cancer therapeutic agents, there is a growing interest in dietary phytochemicals, which also have HDAC inhibitory activity.[8] Natural products offer a fair chance for the design of new HDAC inhibitors.[9] Hence focusing on the natural inhibitors of HDAC could be a promising therapeutic target for treating cancer. The therapeutic potential of natural products is usually accredited to their bioactive constituents commonly flavonoids and phenolic acids.[10] Natural compounds have been identified to play an important role in several cancer preventive mechanisms which include inhibition of oxidation, induction of apoptosis, alteration in the immune system response, and their consequences at the cellular signaling system.[11]

Macrotyloma uniflorum Lam. (Verdc.), commonly known as horse gram, belongs to the family *Fabaceae*. Conventionally, it has been widely utilized for the treatment of various diseases including kidney stones, asthma, leukoderma, urinary discharge, and heart disease.[12] It is a potent source of antioxidant-rich food grain.[13] We investigated the cytotoxic activity of *M. uniflorum* along with an HDACi (SAHA), to evaluate the effects of combination therapy on the human leukemia cancer cell line (HL-60). We hypothesized that the bioactive components present in the seeds of *M. uniflorum* may facilitate the anticancer activity of HDAC inhibitor (SAHA).

MATERIALS AND METHODS

Chemicals

Follin-Ciocalteu reagent (≥98%), gallic acid (≥98%), quercetin (≥95%), SAHA (≥98%), ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) (98%), 4', 6-diamidino-2-phnylindole (DAPI) (≥98%), acridine orange (≥98%), LC-3B, Alexa fluor-488 conjugated secondary antibody were purchased from Sigma-Aldrich. BEZ-235 was obtained from Selleck Chemicals (USA). Fetal Calf Serum, RPMI-1640, pencillin, phosphate-buffered saline (PBS) were procured from HiMedia Laboratories. All the other chemicals and reagents used were of analytical grade.

Fractionated solvent extraction

M. uniflorum seeds were purchased from the local market of Hyderabad (India). The seeds were identified and authenticated by Dr. Md. Salik Noorani, Department of Botany, Jamia Hamdard, New Delhi, India. A voucher specimen (No. DBT-08-06) is deposited in the herbarium of the Department of Pharmacognosy and Phytochemistry, Jamia Hamdard. The extraction of seeds was carried using the protocol by Senawong *et al.*, with slight modifications.[14] Briefly, 50 g of powdered seeds were sequentially macerated with hexane, ethyl acetate, methanol, and water (500 ml each) on a platform shaker for 48 h. The methanol, ethyl acetate, and hexane extracts were oven-dried at 37°C, whereas the obtained aqueous extracts were freeze-dried. Finally, the obtained extracts were weighed, their yield was calculated and stored at-20°C until used. All the solvents used were of high performance liquid chromatography grade purchased from HiMedia Laboratories.

Determination of total phenolic content and total flavonoid content

The Folin Ciocalteau reagent was used to determine the total phenolic content (TPC) of the extracts as previously reported.[15] Briefly, 200 µL of each sample (2 mg/ml) were added to 1 ml of Folin–Ciocalteau reagent and 1 ml of 7.5% w/v sodium carbonate. The absorbance was measured after 2 h at 726 nm and levels of TPC were determined in triplicate. Gallic acid was used as standard and TPC was expressed as gallic acid equivalents in mg/g of dry weight. The concentration of TPC was calculated from the calibration plot (Y =0.0018x–0.0098, R =0.9791).

Total flavonoid content (TFC) was determined using a method based on the formation of a flavonoid-aluminum complex.[16] The absorbance of samples (2 mg/ml) was read at 515 nm using ultraviolet (UV) spectrophotometer and compared with the quercetin calibration curve. Quercetin was utilized as a standard and the TFC of extracts was expressed in milligram quercetin/g extract. The analysis was performed in triplicate and mean values were reported. The concentration of TFC was calculated from the calibration plot (Y =0.6894x +0.0024, R =0.992).

Maintenance of cell line

The leukemia cell line (HL-60) was procured from the European Collection of Cell Cultures (ECACC), UK. The cell line was maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin. Cells were cultured at 37°C in CO_2 incubator (New Brunswick, Galaxy 170R, Eppendorf) with internal atmosphere conditions of 95% humidity and 5% CO_2 gas.

Cytotoxicity assay

MTT assay was used to assess the effect of seed extracts on cell proliferation. The cells were seeded 12 h before drug treatment in 96 well plates at a cell density of 1 × 10^4 cells/well. After 48 h exposure to different concentrations of extracts (ranging from 6.25 µg/ml to 1000 µg/ml), 20 µl of MTT was added to each well and incubated for further 4 h. Media was removed by slowly tapping plates on blotting sheets and then 150 µl DMSO was added to each well. The absorbance was recorded at the wavelength of 570 nm in the microplate reader and cytotoxicity was calculated as follows: % Inhibition = 100 − (Test OD/Non-treated OD) ×100).[17]

Measurement of histone deacetylase activity against HeLa cell nuclear extract

HDAC inhibition was assessed by HDAC fluorometric drug screening kit procured from Biovision (catalog number K340–100). The assay was performed according to the manufacturer's instructions. All the extracts (water, methanol, ethyl acetate, and hexane) at a concentration of 400 µg/ml were screened for HDAC inhibition. In addition, the combination of aqueous extract (400 µg/ml) and SAHA (3.5 µM) was also screened. SAHA was used as a positive control (20 µM). Fluorescence was measured using a Spectra Max Gemini XPS microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA, U. S. A.) with excitation at 360 nm and emission at 460 nm.

Histone deacetylase isoform specificity

HDAC inhibition activity of the aqueous extract (400 µg/ml) along with SAHA (3.5 µM) was evaluated against HDAC isoforms after initial

screening against HeLa nuclear extract. SAHA was used as a positive control (3.5 μM). Fluorometric HDAC isoform inhibition assay kits from BPS biosciences (BPS Bioscience Inc., USA) measured the HDAC isoform specificity of aqueous extract along with SAHA. The kits were used according to instructions from the manufacturer. The fluorescence was observed at 360 nm of excitation wavelength and 460 nm of emission wavelength.

Apoptosis assessment by 4′, 6-diamidino-2-phnylindole staining

DAPI staining was performed following a standard protocol.[18] HL-60 cells were cultured in aqueous extracts of *M. uniflorum* in 24 well plates along with different concentrations of SAHA (0.125 μM, 0.5 μM, 1 μM and 3.5 μM) for 48 h. After incubation the cells were washed in PBS, fixed with 2% paraformaldehyde for 15 min and were treated with 0.2% triton X-100 in PBS for 15 min at room temperature. Cells after washing with PBS were stained with DAPI (1 μg/ml) and incubated in dark for 30 min. SAHA was used as control. The cells were then examined and photographed using a fluorescence microscope.

Detection of DNA damage by the comet assay

The comet assay was performed according to the guidelines proposed by Singh *et al*.[19] The slides were analyzed at ×40 using computerized image analysis system (Komet 5.5). The experiment was performed under dim light to prevent any damage to the DNA that might result from fluorescent white light.

Autophagy detection by orange acridine assay using fluorescence microscopy

Autophagy induction was studied by staining cells with orange acridine dye.[20] HL-60 cells were seeded into six-well plates and treated for 48 h with various concentrations of SAHA (0.125 μM, 1.0 μM, 2.5 μM and 3.5 μM) along with aqueous extract. As a positive control, the BEZ-235 (500nM) was used. The cells were incubated for 15 min with 1 μg/ml acridine orange until the experiment was terminated and incubated at 37°C. The cells were then extracted and washed with PBS before analyzing on a fluorescent microscope (Olympus Fluoview FV 1000) before the examination.

Autophagy detection by immunofluorescence microscope

HL-60 cells were seeded in 6-well plates and treated with aqueous extract (400 μg/ml) for 48 h in the presence of 3.5 μM of SAHA. After the incubation cells were washed twice in PBS and then attached to poly-lysine coated coverslips. The cells were fixed in 4% paraformaldehyde for 15 min at room temperature. Further, the cells were permeabilized for 10 min at room temperature in PBS with 0.1% Triton X-100. To block the nonspecific binding, sites the cells were treated with 10% bovine serum albumin. Cells were then incubated with LC-3B diluted 1:500 in PBS at room temperature for I h. and Alexa Fluor 488 conjugated secondary antibody diluted 1:500 in PBS for 1 h at room temperature. Cells were then washed three times in PBS and stained with DAPI and 1 μg/ml in PBS. The coverslips were placed over glass slides and cells were imagined by a fluorescent microscope (Olympus Fluoview FV1000) by using a ×60 oil immersion objective lens.[21]

Statistical analysis

Statistical differences were determined using one-way analysis of variance (ANOVA) using Graph Pad Prism 8 (GraphPad Software Inc., San Diego, USA). The results are presented as means ± standard deviation (SD) of three independent experiments. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Extraction yields

Extraction yield is defined as the weight percentage of the obtained dried crude extract with respect to the initial amount of the dried powder. Considerable variations in the percentage yields were found among the extracts obtained using different solvents. The highest percentage yield was recorded for water extract (19.71 ± 0.08)), while the lowest yield was observed in the hexane extract (5.46 ± 0.07). The percentage yield in methanol and ethyl acetate was 15.09 ± 0.04 and 8.25 ± 0.02, respectively. The findings of this analysis showed that the nature and polarity of solvent influence the percentage yield of the extract. It was in line with the report by Do *et al*., who reported a high yield in Limnophila aromatic extracts when using polar solvents.[22] Previous studies on other plant species also showed the yield of samples obtained is higher in polar solvents than nonpolar which corroborates the results of this study.[23]

Total phenol and flavonoid content of *Macrotyloma uniflorum* extracts

The results of TPC in *M. uniflorum* extracts are summarized in Table 1. Results reveal significant differences between different extracting solvents ($P < 0.05$). The aqueous extract had the highest TPC (311.21 ± 2 mg gallic acid/g), whereas hexane extract had the lowest TPC (34.33 ± 1.72 mg gallic acid/g). The TPC results indicated that a higher solvent polarity yields higher amounts of phenolic compounds. In other words, results suggested that extraction with low polarity solvents resulted in a lower content of polyphenols.

The results of TFC in *M. uniflorum* extracts are summarized in Table 1. The results showed a similar trend as observed in TPC, with the highest values observed in aqueous extract and lowest values recorded in hexane extract. The flavonoid content varied from 19.96 ± 1.04 to 89.96 ± 4.62 mg Quercetin/g extract [Table 1]. Tests were carried out in triplicate for both the assays. One-way ANOVA results revealed that there were significant differences between the extracts ($P < 0.05$). The TFC is also influenced by the polarities of solvent.

The results of the current study showed that different extracts contained different levels of TPC and TFC. The water extract had significantly higher phenolic and flavonoid content than other solvents. Results of this study were in agreement with a study,[23] which showed that polar solvent extracts were rich in phenolic and flavonoid contents. Flavonoids and phenolic acids are the dominant phenolic compounds present in lentils, black gram, peas, and common beans.[24]

Polyphenols that include flavonoids, phenolic acids, lignans, and stilbenes comprise a diverse group of secondary metabolites abundant in plants, where they play key roles in regulating growth, metabolism, protecting against UV radiation and cancer.[25] Flavonoids and phenolic

Table 1: Percentage extraction yields and the chemical components analyzed from *Macrotyloma uniflorum* seeds using different solvents

Parameters	Water	Methanol	Ethyl acetate	Hexane
Total phenol content (mg GAE/g extract)	311±4.84	273.22±4.32	67.66±3.67	34.33±4.11
Total flavonoid content (mg QE/g extract)	89.96±4.62	87.2±4.21	23.07±4.08	19.96±4.52
Yield (%)	19.71±0.08	15.09±0.04	8.25±0.02	5.46±0.07

acids are the dominant phenolic compounds present in lentils, black gram, peas, and common beans.[26]

In vitro cytotoxic activity of *Macrotyloma uniflorum* extracts

Results revealed no significant cytotoxicity of the extracts toward the HL-60 cell line. However, the extracts along with different concentrations of SAHA (0.5 μM, 1.0 μM, 3.5 μM, and 4.0 μM) showed cytotoxicity. Hexane, ethyl acetate, and methanol extracts proved to be very weak ligands of HDACs, displaying insignificant inhibition against all the cancer cell lines tested. The results indicated that the prominent mode of cell death associated with aqueous extract is due to HDAC inhibition. Aqueous extract at a concentration of 400 μg/ml along with SAHA (3.5 μM) was found to be significantly cytotoxic against HL-60 (100 ± 5). We observed a low concentration of SAHA (3.5 μM) in combination with aqueous extract is required to cause 100% inhibition [Table 2]. Hence, in all the further experiments, the concentration of aqueous extract is taken as 400 μg/ml in combination with SAHA, and the concentration is expressed with respect to SAHA. Cytotoxicity of aqueous extract along with SAHA was evaluated at lower concentrations (0.25–4.5 μM) to determine IC_{50} values against HL-60 cell line. The combination displayed IC_{50} of 0.9 ± 0.2 μM as compared to SAHA alone (1.3 ± 0.2 μM). The values given represent the mean ± SDs of three independent experiments carried out in triplicate.

In vitro histone deacetylase inhibitory activity of the extracts from *Macrotyloma uniflorum* seeds

The effect of different extracts at a concentration of 400 μg/ml of *M. uniflorum* (aqueous, methanol, ethyl acetate, and hexane), and the combination of aqueous extract (400 μg/ml) and SAHA (3.5 μM) on *in vitro* HDAC activity was examined using HeLa nuclear extract as a source of the HDAC enzymes. As shown in [Figure 1], among various polarity extracts tested, the aqueous extract exhibited the most potent HDAC inhibition (61.12% ±2.1%) as compared to the other extracts. Furthermore, the percentage of inhibition by SAHA (77.98% ±2.7%) was enhanced in the presence of aqueous extract (88.28% ±1.0%). The values are the means of three experiments.

After initial screening against HeLa nuclear extract, HDAC inhibition activity of aqueous extract along with SAHA was evaluated against HDAC isoforms. Aqueous extract along with SAHA exhibited interesting HDAC isoform specificity. The combination was found most active against HDAC isoforms 1, 2, 3 and 8 with IC_{50} values 150 ± 0.42 nM, 457 ± 2 nM, 263 ± 0.7 nM, and 289 ± 1.5 nM, respectively [Table 3]. Aqueous extract along with SAHA was found to be moderately active against HDAC isoforms 4 and 5 with IC_{50} values in the micromolar range, while it was found to be least potent against HDAC 9, 10, and 11. These results indicate that the combination of aqueous extract along with SAHA is more active towards class I HDACs as compared to other HDACs.

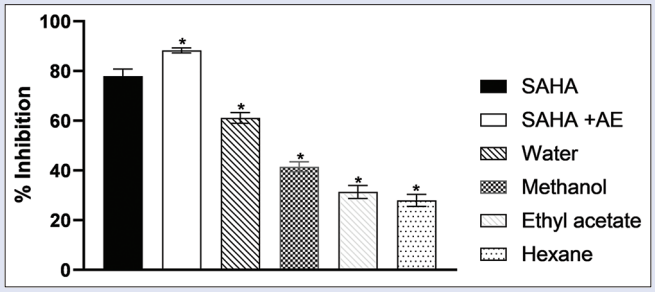


Figure 1: Histone deacetylase inhibition activities of different extracts of *Macrotyloma uniflorum* at a concentration of 400μg/ml against HeLa nuclear extracts. Suberoylanilide hydroxamic acid was used as positive control at a concentration of 20μM concentration. Each value represents the mean ± standard deviation of three experiments, performed in duplicate. Asterisks (*) denote significant differences ($P < 0.05$)

Aqueous extract of *Macrotyloma uniflorum* induces formation of apoptotic bodies

The change in nuclear morphology is an essential marker of cell death. The effect of aqueous extract of *M. uniflorum* on cell death was determined by DAPI staining and fluorescence microscopy. Untreated HL-60 cells appeared round in shape while as nuclei of treated cells showed alteration in morphology including formation of blebs and apoptotic bodies [Figure 2]. The changes in morphology of cells were more prominent at higher concentrations. These results indicate that aqueous extract induces apoptosis.

Macrotyloma uniflorum induced DNA damage

Induction of DNA damage by aqueous extract was studied in HL-60 cells using the comet assay. Aqueous extract was found to induce significant DNA damage in HL-60 [Figure 3]. DNA damage induced by aqueous

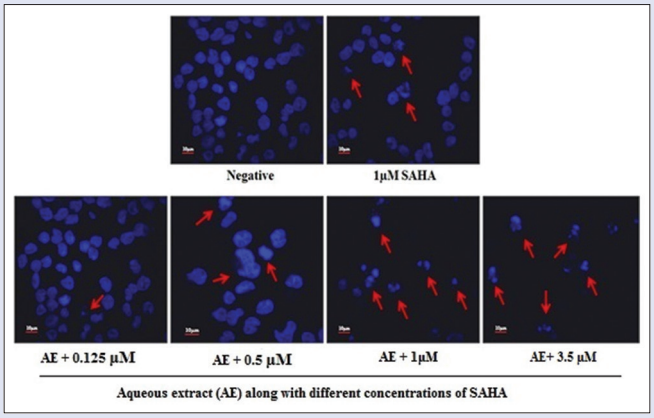


Figure 2: Effect of aqueous extract on nuclear morphology observed under fluorescence microscope (×200, scale bar: 10 μm). The aqueous extract induced the formation of apoptotic bodies. The data are representative of three separate sets of experiments

Table 2: Percent growth inhibition of *Macrotyloma uniflorum* aqueous extract along with different concentrations of SAHA against HL-60 cell line by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

Cancer cell type		HL-60			
Aqueous extract (400μg/ml) along with different concentration of SAHA	Concentration (μM)	4.0	3.5	1.0	0.5
	Inhibition (%)	100±6	100±5	65±4	58±6
SAHA alone	Inhibition (%)	91±4	68±2	50±5	38±2

Each value represents mean±SD of three experiments, performed in duplicate. SAHA:Suberoylanilide Hydroxamic Acid; AE: Aqueous extract; HL: Human leukemia; SD: Standard deviation

Table 3: Histone deacetylase inhibition activities (IC_{50}=nM) of combination of *Macrotyloma uniflorum* seed extract and vorinostat against histone deacetylase isoforms

	Class I				Class IIa			Class IIb	Class IV
	HDAC 1	HDAC 2	HDAC 3	HDAC 8	HDAC 4	HDAC 5	HDAC 9	HDAC 10	HDAC 11
AE + SAHA	151±0.42	457±2	263±0.7	289±1.5	8752±1.2	4232±2	>10,000	>10,000	>10,000
SAHA	160±1.0	467±0.6	271±2	312±0.7	>10,000	2180±2	>10,000	982±1.2	>10,000

The values represent mean±SD of three experiments, performed in duplicate. HDAC: Histone deacetylases; AE: Aqueous extract; SAHA: Suberoylanilide Hydroxamic Acid (vorinostat); SD: Standard deviation

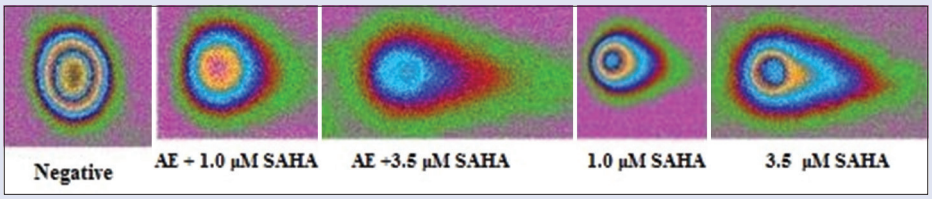


Figure 3: DNA damage induced by *Macrotyloma uniflorum* aqueous extract in HL-60 cells was studied by the comet assay. The length of the tail depicts the damage induced by aqueous extract

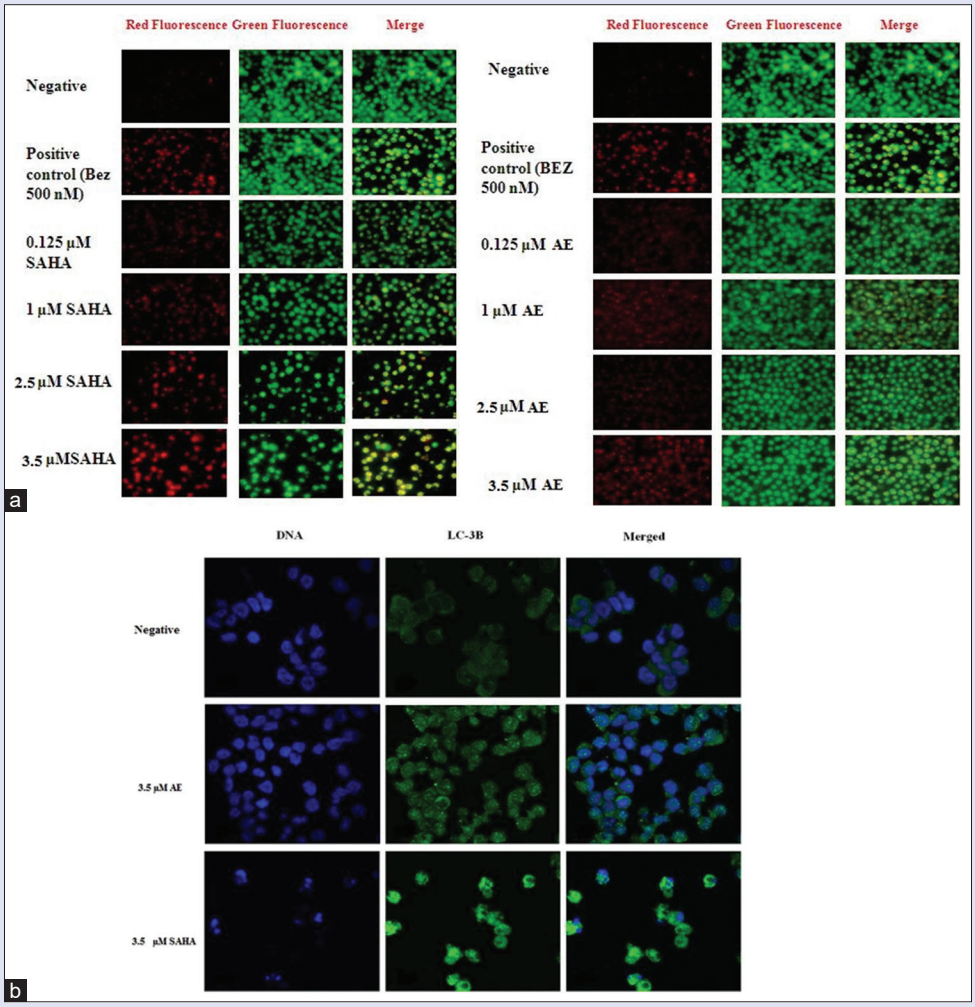


Figure 4: (a) HL-60 cells treated with aqueous extract along with suberoylanilide hydroxamic acid (AE) were stained with acridine orange dye and examined under a fluorescent microscope. It induced the formation of acidic vacuoles in the cytoplasm. (b) HL-60 cells treated with aqueous extract along with suberoylanilide hydroxamic acid were subjected to immunofluorescent staining and examined under a fluorescent microscope. Aqueous extract caused punctuated distribution of LC-3B. (magnification × 100, scale bar: 50 µm)

extract along with SAHA was found to be maximum at 3.5 μM concentration.

Induced autophagy

Generally, autophagy facilitates the suppression of tumors.[27] As with other cytotoxic agents, HDAC inhibitors typically cause autophagy.[28] To investigate whether aqueous extract could induce autophagy, we used acridine orange dye. Interestingly, aqueous extract along with SAHA caused aggregation of autophagic vacuoles in the HL-60 cell cytoplasm [Figure 4]. To further validate autophagy induction by aqueous extract, intracellular distribution of LC-3B was observed by immunofluorescent microscopy [Figure 4a]. HL-60 cells treated with aqueous extract punctuated distribution of LC-3B was seen [Figure 4b], thus, demonstrating that aqueous extract induced autophagy in HL-60 cells.

It has been reported that the general health benefits of pulses are associated with macronutrients; however, there is sufficient evidence to suggest that certain nonnutritional compounds that also play an important role in maintaining human health.[29] These nonnutritional constituents include enzyme inhibitors, phytic acid, lectins, phytosterols, phenolic compounds, and saponins that make pulses suitable for use in a wide range of food products.[30] The food and Agriculture Organization of the United Nations declared 2016 as the International Year of Pulses to highlight the important nutritional properties and the low environmental impact of pulse production worldwide.[31] Experimental, epidemiological, and clinical studies indicate associations between food legume intake and declining cancer incidence.[32,33] Components especially carotenoids, polyphenols, phytosterols, and dietary fibers have been shown to reduce the risk of major human chronic diseases such as cancer, cardiovascular diseases, and Parkinson's disease.[34,35]

The natural bioactive compounds can act in synergy with anticancer drugs.[36] SAHA exerts significant anti-cancer activity in a number of solid and hematological malignancies through increasing the levels of reactive oxygen species, cell apoptosis, differentiation, growth arrest and angiogenesis inhibition.[37] Nihal *et al*., reported that the vorinostat and polyphenolic antioxidant-EGCG (-)-epigallocatechin-3-gallate combination synergistically inhibits the growth of melanoma cells by enhancing apoptosis and activating p21, p27 and caspases.[38] Combination of Vorinostat and tea polyphenols synergistically enhance treatment efficacy and reduce the adverse side effects of anticancer drugs in cancer patients. Our study demonstrated that the combination induced significant inhibition and apoptosis of HL-60 cells. It is characterized by membrane blebbing, cytoplasmic condensation, apoptotic body formation, DNA fragmentation.[39] Further, the results show that the extract of *M. uniflorum* facilitated cell death and autophagy in leukemia. Taken together, the findings of this study provide the scientific evidence for the possibility of combining anticancer drugs and natural products as a promising new combinatorial therapeutic approach against cancer.

CONCLUSION

Our findings demonstrated that the *M. uniflorum* aqueous extract along with SAHA enhanced HDAC inhibitory activity, apoptosis, and autophagy in leukemia cells. *M. uniflorum* possesses HDAC inhibitory activity making it more attractive in combination therapy. The findings of this study provide the scientific evidence for the possibility of combining anticancer drugs and natural products as a promising new combinatorial therapeutic approach against cancer. The results of the present study warrant thorough research on combination therapy with other anticancer drugs. Also, clinical validation of these results is required to assess the anticancer activity and to help validate drug candidates for the treatment.

Acknowledgements

The authors are grateful to the Head, Department of Biotechnology, Jamia Hamdard for providing the necessary facilities to carry out the work. Indian Council of Medical Research, New Delhi is acknowledged for providing Senior Research Fellowship to Azra Anjum.

Financial support and sponsorship

Financial assistance from Indian Council of Medical Research to Azra Anjum (SRF/03/2018/SBHSR) is gratefully acknowledged.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Guo M, Peng Y, Gao A, Du C, Herman JG. Epigenetic heterogeneity in cancer. Biomark Res 2019;7:1-19.
2. Han M, Jia L, Lv W, Wang L, Cui W. Epigenetic enzyme mutations: Role in tumorigenesis and molecular inhibitors. Front Oncol 2019;9:194.
3. Haery L, Thompson RC, Gilmore TD. Histone acetyltransferases and histone deacetylases in B- and T-cell development, physiology and malignancy. Genes Cancer 2015;6:184-213.
4. Suraweera A, O'Byrne KJ, Richard DJ. Combination therapy with histone deacetylase inhibitors (HDACi) for the treatment of cancer: Achieving the full therapeutic potential of HDACi. Front Oncol 2018;8:92.
5. Li Y, Seto E. HDACs and HDAC inhibitors in cancer development and therapy. Cold Spring Harb Perspect Med 2016;6:a026831.
6. Marks PA, Breslow R. Dimethyl sulfoxide to vorinostat: development of this histone deacetylase inhibitor as an anticancer drug. Nat Biotechnol 2007;25:84-90.
7. Eckschlager T, Plch J, Stiborova M, Hrabeta J. Histone deacetylase inhibitors as anticancer drugs. Int J Mol Sci 2017;18:1414.
8. Merarchi M, Sethi G, Shanmugam MK, Fan L, Arfuso F, Ahn KS. Role of natural products in modulating histone deacetylases in cancer. Molecules 2019;24:E1047.
9. Hanikoglu A, Hanikoglu F, Ozben T. Natural product inhibitors of histone deacetylases as new anticancer agents. Curr Protein Pept Sci 2018;19:333-40.
10. Song M, Wu K, Meyerhardt JA, Ogino S, Wang M, Fuchs CS, *et al*. Fiber intake and survival after colorectal cancer diagnosis. JAMA Oncol 2018;4:71-9.
11. Losson H, Schnekenburger M, Dicato M, Diederich M. Natural compound histone deacetylase inhibitors (HDACi): Synergy with inflammatory signaling pathway modulators and clinical applications in cancer. Molecules 2016;21:E1608.
12. Tiwari AK, Manasa K, Kumar DA, Zehra A. Raw horse gram seeds possess more *in vitro* antihyperglycaemic activities and antioxidant properties than their sprouts. Nutrafoods 2013;12:47-54.
13. Anand A, Priyanka U, Madhusudana K, Nayak VL, Zehra A, Babu KS, *et al*. Administration of roasted barley and roasted horse gram powders pacified chronic sucrose-induced dysglycemia and dyslipidemia in rats and exerted *in vitro* potent antioxidative stress effect. Pharmacogn Mag 2018;14:578-90.
14. Senawong T, Misuna S, Khaopha S, Nuchadomrong S, Sawatsitang P, Phaosiri C, *et al*. Histone deacetylase (HDAC) inhibitory and antiproliferative activities of phenolic-rich extracts derived from the rhizome of Hydnophytum formicarum Jack: Sinapinic acid acts as HDAC inhibitor. BMC Complement Altern Med 2013;13:232.
15. Menichini G, Alfano C, Provenzano E, Marrelli M, Statti GA, Somma F, *et al*. Figure latex (*Ficus carica* L. cultivar Dottato) in Combination with UV irradiation decreases the viability of A375 melanoma cells *in vitro* . Anticancer Agents Med Chem 2012;12:959-65.
16. Marrelli M, Menichini F, Conforti F. A comparative study of *Zingiber officinale* Roscoe pulp and peel: Phytochemical composition and evaluation of antitumour activity. Nat Prod Res 2015;29:2045-9.
17. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55-63.
18. Radhika NK, Sreejith PS, Asha VV. Cytotoxic and apoptotic activity of *Cheilanthes farinosa* (Forsk.) Kaulf. Against human hepatoma, Hep3B cells. J Ethnopharmacol 2010;128:166-71.
19. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 1988;175:184-91.

20. Kim JY, Cho TJ, Woo BH, Choi KU, Lee CH, Ryu MH, *et al.* Curcumin-induced autophagy contributes to the decreased survival of oral cancer cells. Arch Oral Biol 2012;57:1018-25.
21. Ait-Mohamed O, Battisti V, Joliot V, Fritsch L, Pontis J, Medjkane S, *et al.* Acetonic extract of *Buxus sempervirens* induces cell cycle arrest, apoptosis and autophagy in breast cancer cells. PLoS One 2011;6:e24537.
22. Do QD, Angkawijaya AE, Tran-Nguyen PL, Huynh LH, Soetaredjo FE, Ismadji S, *et al.* Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of *Limnophila aromatica*. J Food Drug Anal 2014;22:296-302.
23. Nguyen VT, Pham NM, Van Vuong Q, Bowyer MC, van Altena IA, Scarlett CJ. Phytochemical retention and antioxidant capacity of xao tam phan (*Paramignya trimera*) root as prepared by different drying methods. Dry Technol 2016;34:324-34.
24. Xu BJ, Chang SK. A comparative study on phenolic profiles and antioxidant activities of legumes as affected by extraction solvents. J Food Sci 2007;72:S159-66.
25. Scalbert A, Manach C, Morand C, Rémésy C, Jiménez L. Dietary polyphenols and the prevention of diseases. Crit Rev Food Sci Nutr 2005;45:287-306.
26. Ombra MN, d'Acierno A, Nazzaro F, Riccardi R, Spigno P, Zaccardelli M, *et al.* Phenolic composition and antioxidant and antiproliferative activities of the extracts of twelve common bean (*Phaseolus vulgaris* L.) endemic ecotypes of southern italy before and after cooking. Oxid Med Cell Longev 2016;2016:1-12.
27. Santana-Codina N, Mancias JD, Kimmelman AC. The role of autophagy in cancer. Annu Rev Cancer Biol 2017;1:19-39.
28. Rikiishi H. Autophagic and apoptotic effects of HDAC inhibitors on cancer cells. J Biomed Biotechnol 2011;2011:830260.
29. Wang Y, Wang Z, Fu L, Chen Y, Fang J. Legume consumption and colorectal adenoma risk: A meta-analysis of observational studies. PLoS One 2013;8:e67335.
30. Tiwari AK, Swapna M, Ayesha SB, Zehra A, Agawane SB, Madhusudana K. Identification of proglycemic and antihyperglycemic activity in antioxidant rich fraction of some common food grains. Int Food Res J 2011;18:915-23.
31. FAO. Food and Agriculture Organization of United Nations. United Nations: Food Agric Organ; 2016. Available from: http://faostat.fao.org. [Last accessed on 2021 Feb 05].
32. Mathers JC. Pulses and carcinogenesis: Potential for the prevention of colon, breast and other cancers. Br J Nutr 2002;88 Suppl 3:S273-9.
33. Tiwari A, Abhinay B, Babu K, Kumar D, Zehra A, Madhusudana K. Pigeon pea seed husks as potent natural resource of antioxidant and anti-hyperglycaemic activity. Int J Green Pharm 2013;7:252.
34. Mahadevappa R, Kwok HF. Phytochemicals – A novel and prominent source of anti-cancer drugs against colorectal cancer. Comb Chem High Throughput Screen 2017;20:376-94.
35. Xue K, Gu JJ, Zhang Q, Mavis C, Hernandez-Ilizaliturri FJ, Czuczman MS, *et al.* Vorinostat, a histone deacetylase (HDAC) inhibitor, promotes cell cycle arrest and re-sensitizes rituximab- and chemo-resistant lymphoma cells to chemotherapy agents. J Cancer Res Clin Oncol 2016;142:379-87.
36. Jakubowicz-Gil J, Langner E, Bądziul D, Wertel I, Rzeski W. Quercetin and sorafenib as a novel and effective couple in programmed cell death induction in human gliomas. Neurotox Res 2014;26:64-77.
37. Apuri S, Sokol L. An overview of investigational Histone deacetylase inhibitors (HDACis) for the treatment of non-Hodgkin's lymphoma. Expert Opin Investig Drugs 2016;25:687-96.
38. Nihal M, Roelke CT, Wood GS. Anti-melanoma effects of vorinostat in combination with polyphenolic antioxidant (-)-epigallocatechin-3-gallate (EGCG). Pharm Res 2010;27:1103-14.
39. Stewart BW. Mechanisms of apoptosis: Integration of genetic, biochemical and cellular indicators. J Natl Cancer Inst1994;86:1286-96.