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Green Synthesized Zinc Oxide Nanoparticles induced Apoptosis in Human Cervical (HeLa) and Breast (MCF-7) Cancer Cell Lines

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Chemotherapeutic resistance is currently one of the most prevalent problems due to the low absorption and undesirable toxicity of existing medications. Furthermore, non-specific targeting of existing medications is leading to the investigation of novel drug manufacturing strategies to eradicate chronic disorders. Present studies were performed to explore the anti-proliferative potential of synthesized zinc oxide nanoparticles (ZnO NPs) in human breast (MCF-7), human lung (A549) and human cervical (HeLa) cancer cell lines. The ZnO NPs were synthesized by using soybean seed extract. The ZnO NPs were characterized by ultraviolet-visible (UV-Vis) spectroscopy, field-emission scanning electron microscope (FESEM) and X-ray diffraction (XRD). The phytochemicals present in the soybean seed extract was evaluated using gas chromatography-mass spectroscopy (GC-MS) technique. Cytotoxicity effects of ZnO NPs, levofloxacin (LVX) and a combination of ZnO NPs-LVX against MCF-7, A549 and HeLa cancer cell lines was evaluated by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay. Annexin assays were performed to explore the apoptosis-inducing potential of ZnO NPs and its drug combination. This showed that tested compounds have potent cytotoxic effects dose-dependently against cancer cell lines. The potent anticancer potential of ZnO NPs-LVX is expressed to utilize in the near future as novel therapeutic strategies.

Keywords: Zinc oxide nanoparticles, Soyabean, Antiproliferative activity, Cervical acitivity, Apoptosis, Annexin V-FITC assays.

INTRODUCTION

Chemotherapeutic medications are widely employed to treat a range of cancers. The considerable side effects of these drugs necessitated the development of contemporary chemotherapy therapies [1,2]. Scientists have known for ages that metallic nanoparticles have excellent antitumor effects [3-5]. Numerous researches showed that nanoparticles, notably metal ones have unique physico-chemical features, which provide a new alternative for treating a range of diseases [6-8]. Since it now has such a wide range of uses, nanotechnology has evolved in a variety of ways [9] and the high surface-to-volume ratio makes them potent biological agents [10].

For the production of nanoparticles, the contemporary biological procedures that are environmentally friendly and cost-effective have been developed to replace the older technologies [11,12]. Environmentally friendly reducing agents, a system of environmentally friendly solvents and environmentally friendly stabilizing agents were used to accomplish this [13,14]. The nanoparticles can be produced by a wide range of medicinal plants and a crucial stage in the biogenesis of nanoparticles is the reduction of metal ions. Additionally, it eliminates the

negative environmental effects of biological synthesis while enabling the production of suitable nanoparticles [13,14]. According to recent studies, green metal nanoparticles are utilized to deliver bioactive compounds and medications to enhance cancer treatment [15,16].

Zinc oxide (ZnO) is well known important chemical in recent years due to its properties [17-19]. Moreover, ZnO is considered as a non-toxic inorganic material having several uses in numerous industries [20]. Biosynthesized ZnO nanoparticles (ZnO NPs) showed promising antioxidative, anti-bacterial and anticancerous properties [21,22]. According to the recent studies, biological syntheses of ZnO NPs can benefit from the anticancer capabilities of medicinal plants [23]. *Glycine max* (soybean) belongs to the family of the Leguminosae, which can grow up to 1.5 m height widely distributed in tropical, subtropical and temperate regions [24]. Soybeans are oil seeds consisting of 20% oil content that have been used, either as food or raw material to extract phytochemicals [24]. Nowadays, it has much importance due to its high protein and lipid content as well as other important nutrients including vitamins, minerals and fatty acids. From a medical perspective, soybean is quite important [25]. Proteins, isoflavones and phytoestrogens are abundant in soybean seeds. Genistein, a soy isoflavone, has also been shown to have anti-osteoporosis, anticancerous, antioxidant and anti-inflammatory actions. It is being explored as a potential drug for the treatment of metabolic diseases [26]. In current study, the biosynthesis of ZnO nanoparticles from soybean seed extract was prepared. The synthesized nanoparticles have been characterized by UV, XRD, field-emission scanning electron microscope (FESEM) and their antiproliferative and apoptosis-inducing potential were evaluated against various cancer cell lines.

EXPERIMENTAL

Zinc acetate dihydrate [$(CH_3CO_2)_2Zn{\cdot}2H_2O$)] from Merck & Co. (Germany) and used as such. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), hydrolyzate, dimethyl sulfoxide (DMSO), 4-(dimethylamino)benzaldehyde, Ehrlich solution and borax-sulfuric acid mixture, dulbazolic mixture modified eagle medium (DMED), bovine serum, antimycotic antibiotic solution were purchased from Sigma-Aldrich, USA. All other chemicals were used are analytical grade. At ambient temperature, distilled water was employed for the preparation of aqueous solutions.

Extract preparation: The soybean seeds were obtained from the local market of Ambala, India. The plant's seed was thoroughly cleaned with distilled water and then air-dried. Using a mixer grinder, the dry seeds were ground into a fine powder. A 0.5 g of finely powdered plant's seed was in kept in 50 mL of double-distilled water for 40 min at 60 ºC. After heating, the plant extract was centrifuged to remove foreign debris and vacuum filtered to achieve a clear plant extract.

GC-MS analysis: The *G. max seeds* aqueous extract was examined using GC-MS (Shimadzu GC 17A, Japan). For this, 1 µL of sample was injected at a 20:1 split ratio and flow rate of helium gas (99.9%) was used at 1 µL/min at 250 ºC (injector) with electron impact (EI) mode with 70 eV ionization energy. The column oven temperature (50 ºC), increased to 280 ºC at 10 ºC/min and finally raised to 300 ºC for 10 min. Comparison of the collected spectral configurations to open access available mass spectral databases (NIST and WILEY libraries) allowed the chemicals to be identified. The mass spectra of the specific unknown chemicals and the known chemicals stored in the software database Libraries were compared. The names, molecular weights and structures of the test materials' constituents were also identified.

Synthesis of ZnO nanoparticles: Zinc acetate (0.1 M) was employed as a precursor in the synthesis and was reduced from Zn^{2+} to Zn ions in 10 mL of soybean seed plant extract at 60 ºC for 10 min. A visible colour shift was used to monitor the reduction of zinc acetate. After the reduction procedure, ZnO nanoparticles were synthesized. The nanoparticles were spun at 10,000 rpm for 15 min at room temperature. The pellet was collected, dried for 3 h and stored in airtight vials for future use.

Characterization: The colour change is the first preliminary test for the synthesis of ZnO nanoparticles. The optical parameter was analyzed by UV-VIS spectroscopy in the range of 280-700 nm. The morphology of ZnO nanoparticles was examined using FESEM and crystallinity was observed using XRD studies.

Cell cultures: Human cervical (HeLa), human breast (MCF-7) and human lung (A549) cell lines were procured from the National Centre for Cell Science (Pune, India) and DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotic antimycotic solution poured into cell culture flasks with cells and incubated at 37 ºC and 5% CO_2.

Anti-proliferative activity

MTT assay: ZnO nanoparticles, levofloxacin (LVX) and a combination of ZnO NPs-LVX were evaluated for their potential to reduced cell proliferation in HeLa, MCF-7 and A549 cells using MTT assay [27]. Different cell lines were seeded (8×10^3 cells/well in 96-well plate) and grown at confluency followed by treating with various doses of the samples (15.62-500 µg/mL) for 24 h. After that MTT (20 µL) was added to each well and plates were stored for 4 h in CO_2 (5%) incubator. Thereafter, the supernatant was discarded and added DMSO (0.1 mL) to solubilize formazan and then the values were recorded at 570 nm.

$$\text{Inhibition of growth (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

where A_0 is the absorbance of control (untreated cells); A_1 is the absorbance of cells treated with different concentrations of the tested samples.

Annexin V-fluorescein isothiocyanate (FITC) assay: Annexin V-FITC apoptosis detection kit procured from BD Pharmingen, San Jose, USA was used for the detection of apoptosis and manufacturer's protocol (BD Pharmingen™ Technical Data Sheet, n.d.) was followed. The HeLa and MCF-7 cells (4×10^5 cells/well) were seeded in six-well plates at confluency and after that cells were treated with different concentration of sample for 24 h. After that cells were harvested by using 1 ×

binding buffer (1 mL) with centrifuged at 2500 rpm for 5 min followed by discarding supernatant and addition of 1 × binding buffer (100 µL), Annexin V-FITC (5 µL) and propidium iodide (5 µL) to the pellet and incubated for 15 min in dark. The BD C6 Accuri flow cytometer was used to analyze the samples.

Statistical analysis: The all studies were carried out in three replicates and the results were presented as Mean ± SE. Means were separated by one-way ANOVA test at 0.5 significant ($p < 0.05$) using Assistat software. Tukey's HSD test was used to determine the statistical differences between test samples.

RESULTS AND DISCUSSION

UV-visible studies: The UV-Vis spectrum of the biosynthesized ZnO NPs prepared from *G. max* extract is depicted in Fig. 1. The biosynthetic ZnO NPs had maxima at 330 nm. Earlier studies on biosynthesized ZnONP nanoparticles have found results consistent with the present study [28]. ZnO NPs have a spherical shape (Fig. 2), as indicated by FE-SEM and

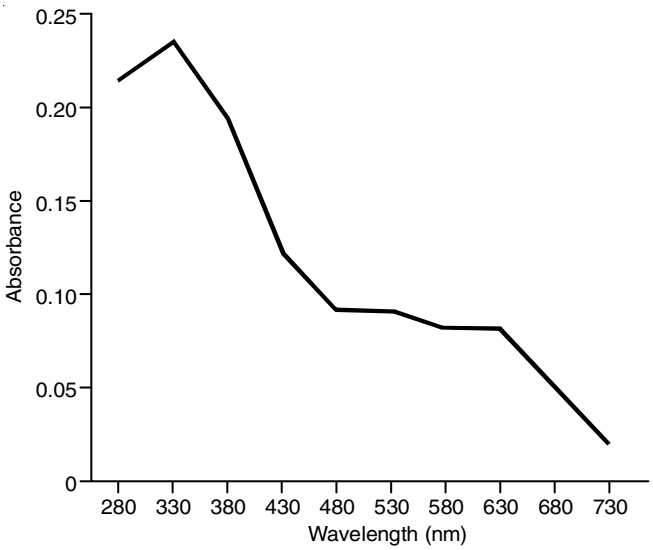

Fig. 1. UV spectrum of ZnO nanoparticles

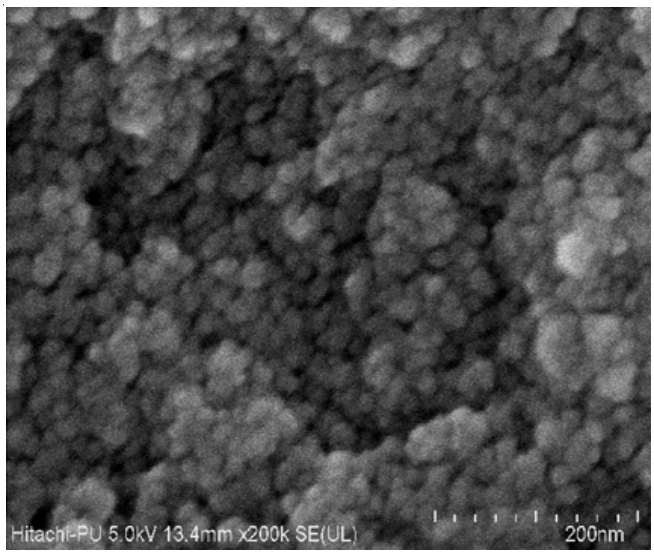

Fig. 2. FESEM of ZnO nanoparticles

its uniformity, well-dispersion and homogeneity were also confirmed. The XRD spectrum (Fig. 3) also provide the details regarding crystallinity of the freshly prepared ZnO NPs using seeds of *Glycine max.* The size of nanoparticles was calculated using Debye-Scherrer equation:

$$D = \frac{K\lambda}{\beta \cos\theta}$$

where D is the particle size in nm; λ is the X-ray wavelength; β is the FWHM value; θ is the Bragg's angle of reflection.

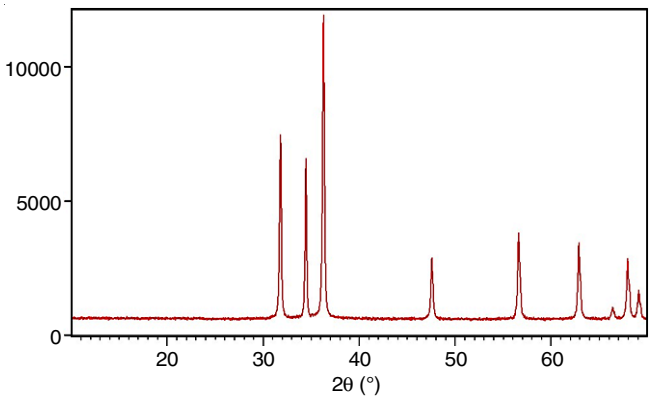

Fig. 3. XRD spectra of ZnO NPs

MTT assay: As per results, ZnO NPs-LVX showed an effective antiproliferative potential against cervical (HeLa) cell line with the lowest GI_{50} value 52.71 µg/mL as shown in Table-1. Hence, the results indicated that ZnO NPs-LVX showed toxicity toward cancer cell lines with minimum GI_{50} value. Flow cytometric tests were performed on the HeLa and MCF-7 cell lines to determine the apoptosis-inducing capacity of the ZnO NPs-LVX.

TABLE-1
GROWTH INHIBITORY CONCENTRATIONS OF SAMPLES AGAINST BREAST (MCF-7), LUNGS (A549) AND CERVIX (HeLa) CANCER CELLS

Sample name	GI_{50} (µg/mL)		
	MCF-7	A549	HeLa
ZnONPs-LVX	58.3	106.58	52.71
ZnONPs	137.88	134.31	67.12
LVX	107.86	127.87	126.51

Annexin V-FITC assays: Apoptosis detection using a flow cytometer in ZnO NPs-LVX treated HeLa and MCF-7 cells was investigated. Fig. 4a-b shows the cell population are in early and late apoptotic increased in ZnO NPs-LVX treated HeLa cells in comparison to untreated cells. At 52.71 µg/mL concentration, early apoptosis (LA) is more (38.5%) whereas in untreated cells (1.7%). The results showed that ZnO NPs-LVX could inhibit the proliferation of HeLa cells *via* apoptosis induction. In histogram, the percentage of living, early apoptotic (EA), late apoptotic (LA) and necrotic cells is displayed.

Similarly, Fig. 5a-b showed ZnO NPs-LVX treated MCF-7 increased early and late apoptotic cell population as compared to untreated cells. At concentration (58.30 µg/mL), early apoptosis (EA) is more (60.7%) as compared to untreated cells (1.6%).

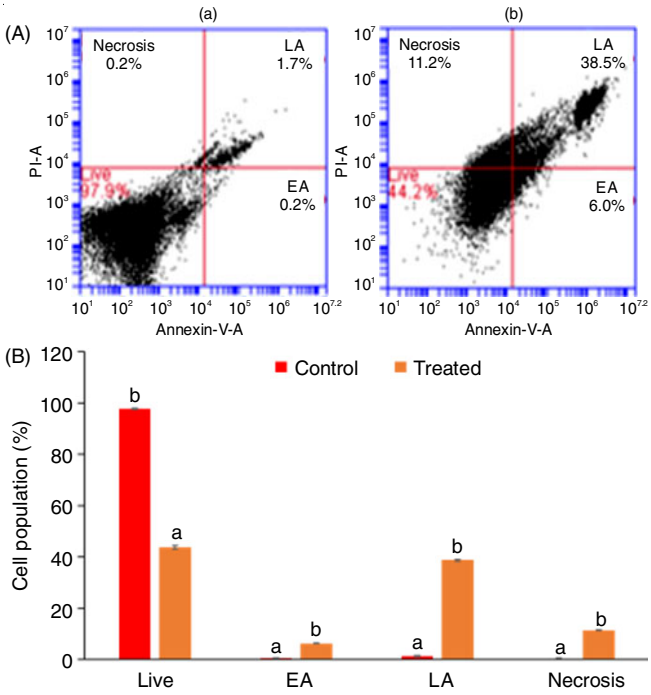

Fig. 4. Apoptosis induced by ZnONPs-LVX in HeLa cells. (A) HeLa cells were treated with 52.71 µg/mL of ZnONPs-LVX for 24 h. Then cells were stained with FITC-conjugated Annexin V and PI for Flow cytometric analysis. (B) Histogram showing the percentage of live, early apoptotic (EA), late apoptotic (LA) and necrotic cells. Where (a) Untreated cells, (b) ZnONPs-LVX treated cells. Significant differences in the data are represented by labels with different letters

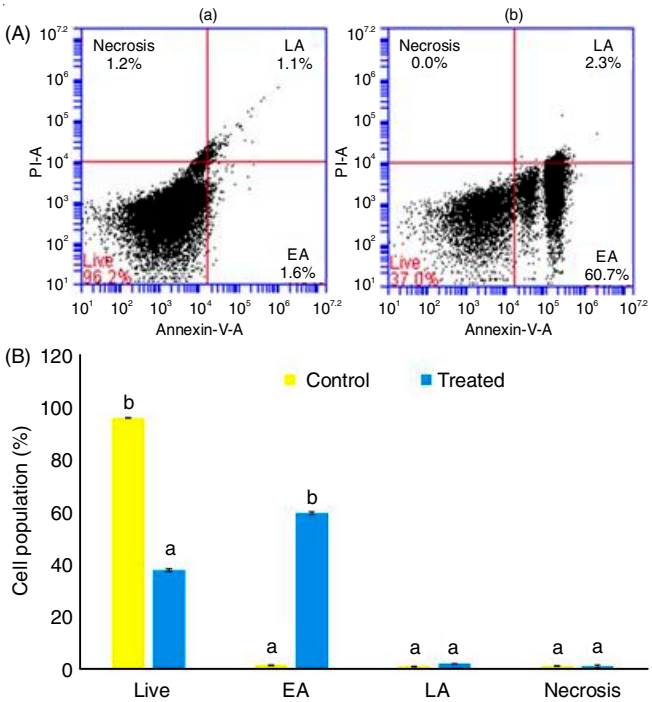

Fig. 5. Apoptosis induced by ZnONPs-LVX in MCF-7 cells. (A) MCF-7 cells were treated with 58.30 µg/mL of ZnONPs-LVX for 24 h. Then cells were stained with FITC-conjugated Annexin V and PI for Flow cytometric analysis. (B) Histogram showing the percentage of live, early apoptotic (EA), late apoptotic (LA) and necrotic cells. Where (a) Untreated cells, (b) ZnONPs-LVX treated cells. Significant differences in the data are represented by labels with different letters

The results indicated that ZnO NPs-LVX has potential to reduce the growth of MCF-7 cells through the induction of apoptosis.

GC-MS studies: Total 32 peaks were showed in aqueous extract of *G. max* seeds by the GC-MS spectrum as shown in Fig. 6. The bioactive compounds present in *G. max* presented in the GC-MS analysis are shown in Table-2 with their retention time (RT) and molecular formula, *etc*. Related studies had shown that the ethanolic extract of *G. max* showed 49 peaks *via* GC-MS analysis [29]. Controlling a nanoparticle's form during synthesis is crucial since it can have an impact on the particle's properties [30].

TABLE-2
LIST OF PHYTOCONSTITUENTS PRESENT IN THE AQUEOUS EXTRACT OF *G. max* SEEDS BY GC-MS ANALYSIS

RT	Compound names	m.f.
3.05	3-Penten-2-one, 4-methyl	$C_6H_{10}O$
3.21	L-Galactose, 6-deoxy	$C_6H_{12}O_5$
3.50	2-Pentanone, 4-hydroxy-4-methyl	$C_6H_{12}O_2$
3.74	Butanoic acid, 2-methyl	$C_5H_{10}O_2$
3.92	*N*-Ethylidene *t*-butylamine	$C_6H_{13}N$
4.91	Methylamine, N-(1-propylpentylidene)	$C_9H_{19}N$
6.43	6-Acetyl-α-*d*-mannose	$C_8H_{14}O_7$
6.50	Z-10-Tetradecen-1-ol acetate	$C_{16}H_{30}O_2$
7.26	Z-8-Methyl-9-tetradecenoic acid	$C_{15}H_{28}O_2$
7.33	Aspidospermidin-17-ol	$C_{23}H_{30}N_2O_5$
7.98	3-Octadecene, (*E*)-	$C_{18}H_{36}$
8.04	Pentadecane	$C_{15}H_{32}$
8.66	1-Hexadecanol, 2-methyl	$C_{17}H_{36}O$
8.71	Aspidospermidin-17-ol	$C_{23}H_{30}N_2O_5$
9.11	2-Methyl-Z-4-tetradecene	$C_{15}H_{30}$
9.31	4-Trifluoroacetoxypentadecane	$C_{17}H_{31}F_3O_2$
9.86	Erucic acid	$C_{22}H_{42}O_2$
9.97	α-D-Glucopyranoside	$C_{18}H_{32}O_{16}$
10.12	1-Hexadecanol, 2-methyl	$C_{17}H_{36}O$
10.28	Dodecanoic acid, 3-hydroxy	$C_{12}H_{24}O_3$
10.61	Octatriacontyl pentafluoropropionate	$C_{41}H_{77}F_5O_2$
10.69	Heneicosane, 11-(1-ethylpropyl)	$C_{26}H_{54}$
11.32	*tert*-Hexadecanethiol	$C_{16}H_{34}S$
11.40	Octatriacontyl pentafluoropropionate	$C_{41}H_{77}F_5O_2$
12.06	Octadecanoic acid	$C_{18}H_{36}O_2$
12.24	*cis*-10-Nonadecenoic acid	$C_{19}H_{36}O_2$
13.44	*cis*-11-Eicosenoic acid	$C_{20}H_{38}O_2$
14.29	12-Methyl-E,E-2,13-octadecadien-1-ol	$C_{19}H_{36}O$
14.34	*cis*-Vaccenic acid	$C_{18}H_{34}O_2$
15.11	Ethanol, 2-(octadecyloxy)	$C_{20}H_{42}O_2$
15.21	Eicosane, 7-hexyl	$C_{26}H_{54}$
15.27	Tetracosane	$C_{24}H_{50}$
25.95	Tetratriacontyl heptafluorobutyrate	$C_{38}H_{69}F_7O_2$
26.20	Oleic acid, eicosyl ester	$C_{38}H_{74}O_2$
26.77	9-Hexadecenoic acid, octadecyl ester, (Z)	$C_{34}H_{66}O_2$
27.51	Nonahexacontanoic acid	$C_{69}H_{138}O_2$

The green synthesis of ZnO-NPs were expanded in the last decade and plant extracts and its isolated constituents have been successfully used to biosynthesize metal nanoparticles [31]. In the present study, the antiproliferative and apoptotic potential of ZnO NPs synthesized from G. *max* seed extract against MCF-7, A549 and HeLa cells using various *in vitro* assays were observed.

The anticancer results showed that HeLa cancer cells are more vulnerable to ZnO NPs as compared to other cell lines

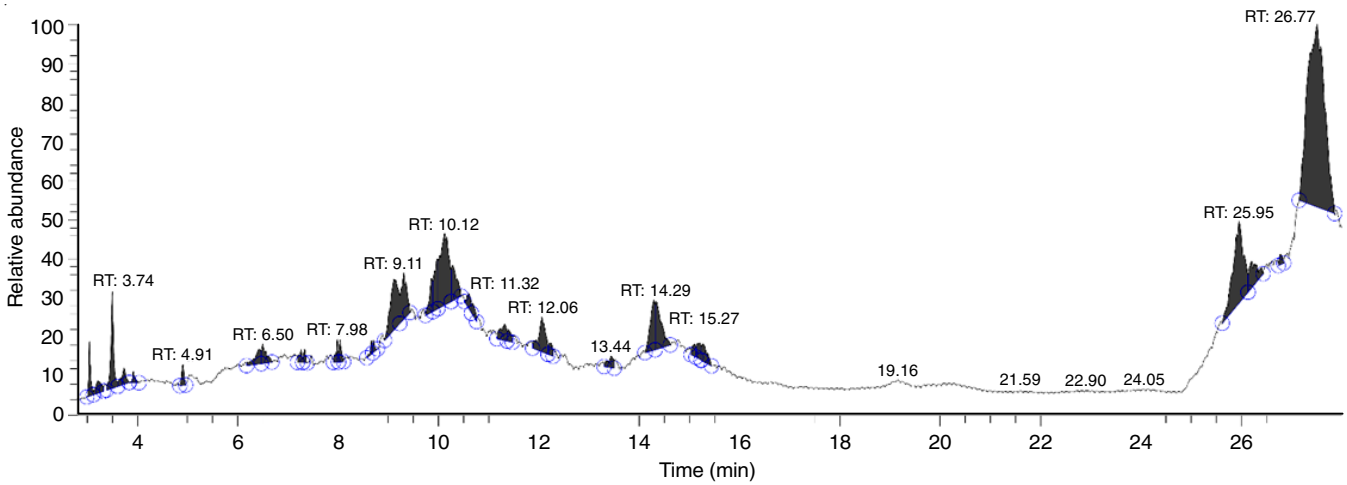

Fig. 6. A typical GC-MS profile of aqueous extract of *G. max* seeds

[32]. Previous research also revealed that the cancer cells are more susceptible to ZnO NPs than normal cells which induced apoptosis in cells through the generation of reactive oxygen species [33]. In flow cytometric studies, ZnO NPs-LVX dose-dependently reduced the growth of Human breast (MCF-7) and HeLa cancer cell line and induces apoptosis. Reactive oxygen species were said to be produced as a result of nanoparticle interactions with macromolecules like proteins and DNA, which led to the oxidative stress and apoptosis induction [34]. Annexin V-FITC-PI staining was used to check the apoptosis-inducing potential of ZnO NPs-LVX against HeLa and MCF-7 cell line using flow cytometer. The data analysis revealed that untreated control Hela cells showed 97.9% live cells, 0.2% early apoptotic, 1.7% late apoptotic cells and 0.2% necrotic cells whereas ZnO NPs-LVX (52.71 µg/mL) treatment in HeLa cells for 24 h showed 44.2% live cells, 6% early apoptosis, 38.5% late apoptosis, 11.2% cells in necrotic quadrant (Fig. 4). Similarly, untreated control MCF-7 cells showed 96.2% live cells, 1.6% early apoptotic, 1.1% late apoptotic cells and 1.2% necrotic cells whereas ZnO NPs-LVX (58.30 µg/mL) treatment in MCF-7 cells for 24 h showed 37.0% live cells, 60.7% early apoptosis and 2.3% late apoptosis as shown in Fig. 5.

Conclusion

The present work highlighted the green synthesis of ZnO nanoparticles from the *Glycine max* seed extract. The uniformity of ZnO NPs was demonstrated by UV-vis, FESEM, and XRD techniques. The ZnO NPs showed the best antiproliferative and apoptosis-inducing potential against HeLa cell lines. Aqueous fraction from G. max has rich in phytoconstituents that might be responsible for the synthesis of nanoparticles. *G. max* and its isolated compounds need to be evaluated as anticancer agents against cervical cancer in the future. Finally, more *in vitro* and *in vivo* research is required before ZnO NPs may be used in clinical settings as an effective anticancer drug.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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