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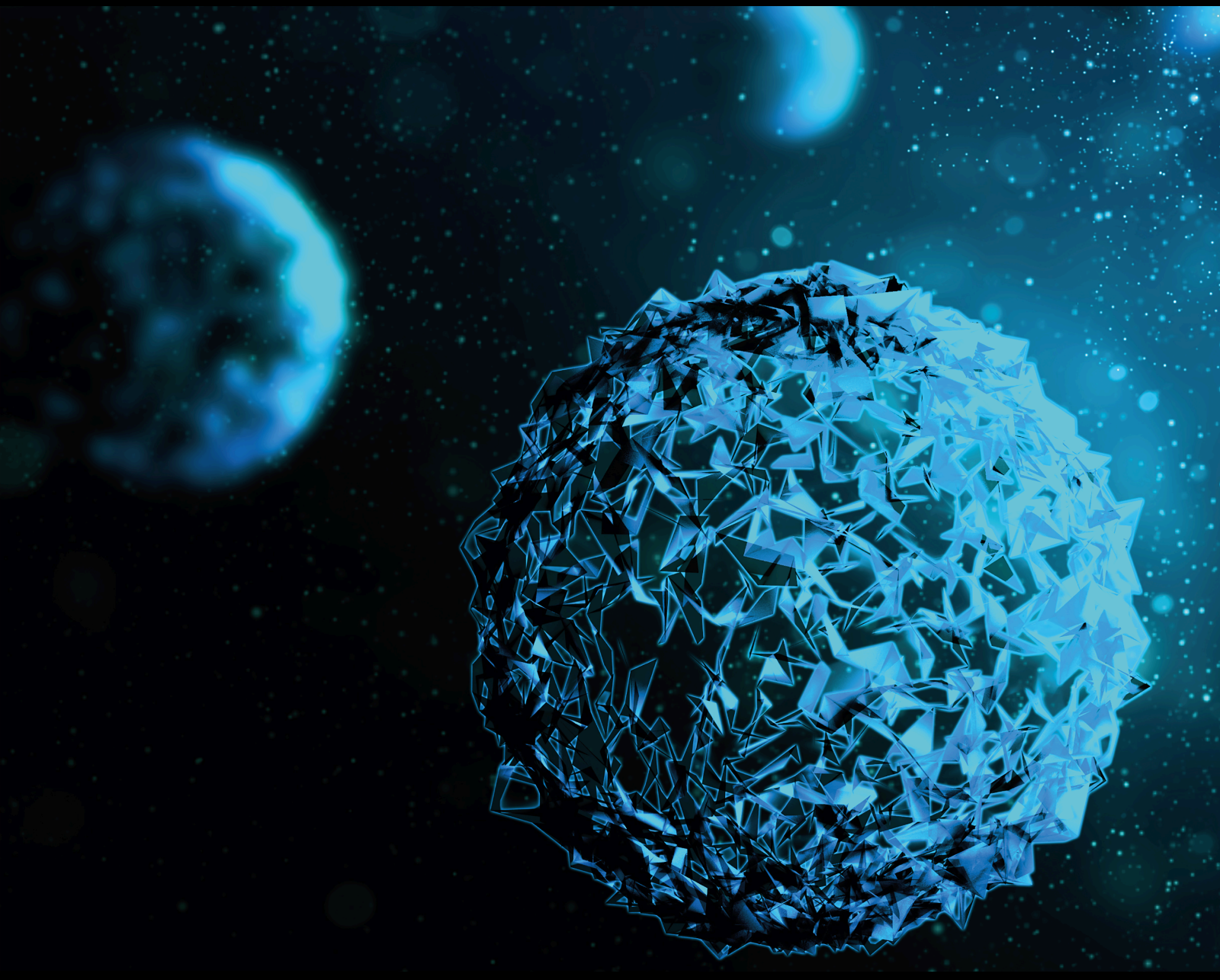
Plant-Derived Anticancer Agents as an Option for Cancer Treatment 2021

Lead Guest Editor: Yearul Kabir
Guest Editors: Meher Un Nessa, Mohammad Nazim, and Chen-Huan Yu



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Contents

Hepatoprotective Screening of *Seriphidium kurramense* (Qazilb.) Y.R. Ling

Maroof Ali, Hidayat Hussain, Amjad Hussain, Abdur Rauf, Wahid Hussain, Manzoor Ullah, Safdar Abbas, Yahya S. Al-Awthan, Omar Bahattab, Muhammad Khan, Ahmed Olatunde, Zainab M. Almarhoon, Yahia N. Mabkhot, Mohammed M. Alshehri, Sevgi Durna Daştan, Mohamed Fawzy Ramadan, and Javad Sharifi-Rad

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Research Article

Natural Compounds or Their Derivatives against Breast Cancer: A Computational Study

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Background. Breast cancer is one of the most common types of cancer diagnosed and the second leading cause of death among women. Breast cancer susceptibility proteins of type 1 and 2 are human tumor suppressor genes. Genetic variations/mutations in these two genes lead to overexpression of human breast tumor suppressor genes (e.g., BRCA1, BRCA2), which triggers uncontrolled duplication of cells in humans. In addition, multidrug resistance protein 1 (MDR1), an important cell membrane protein that pumps many foreign substances from cells, is also responsible for developing resistance to cancer chemotherapy. *Aim of the Study*. The aim of this study was to analyze some natural compounds or their derivatives as part of the development of strong inhibitors for breast cancer. *Methodology*. Molecular docking studies were performed using compounds known in the literature to be effective against BRCA1 and BRCA2 and MDR1, with positive control being 5-fluorouracil, an antineoplastic drug as a positive control. *Results*. The binding affinity of the compounds was analyzed, and it was observed that they had a better binding affinity for the target proteins than the standard drug 5-fluorouracil. Among the compounds analyzed, α-hederin, andrographolide, apigenin, asiatic acid, auricular acid, sinularin, curcumin, citrinin, hispolon, nerol, phytol, retinol palmitate, and sclareol showed the best binding affinity energy to the BRCA1, BRCA2, and MDR1 proteins, respectively. *Conclusions*. α-Hederin, andrographolide, apigenin, asiatic acid, auricular acid, hispolon, sclareol, curcumin, citrinin, and sinularin or their derivatives can be a good source of anticancer agents in breast cancer.

1. Introduction

Breast cancer is one of the common types of diagnosed cancers and the second prime cause of death among women in western countries [1] and develops from breast tissue [2]. Most of the breast cancers are sporadic (90-95%); between 5 and 10% can be attributed to genetic predisposition with patients having a strong family history of the disease [3, 4].

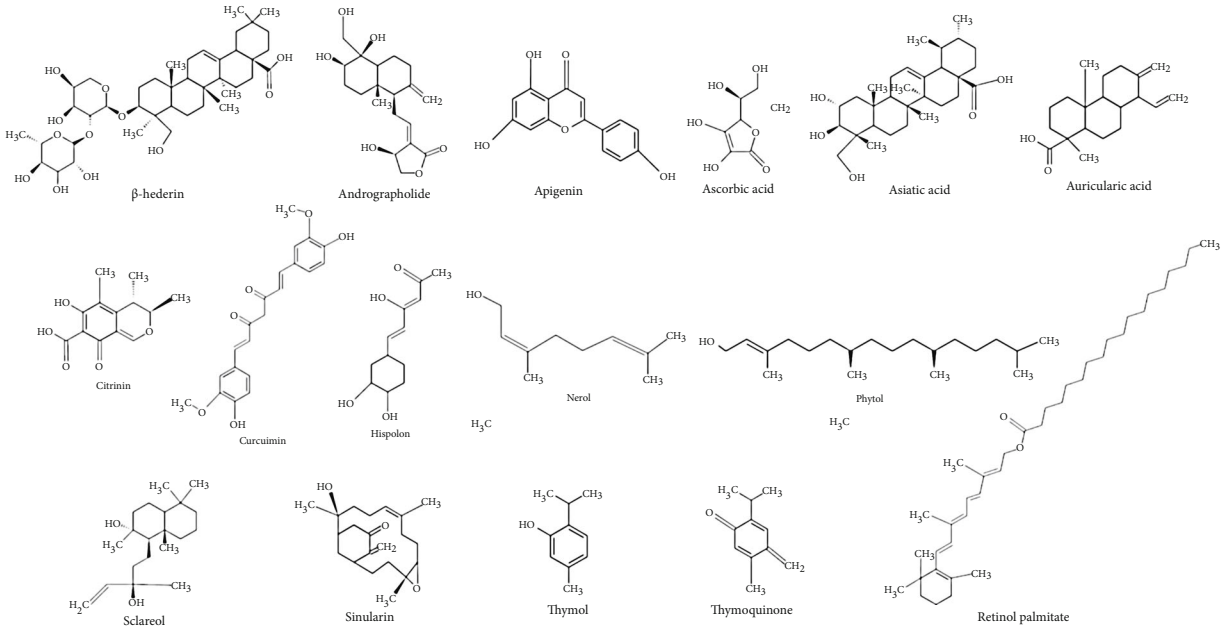


Figure 1: The chemical structure of the screened compounds.

These hereditary breast cancers, a large part about 80-90% cases, are related to germ line mutations within the *BRCA1*, *BRCA2*, and *MDR1* gene [5–9]. By DNA repairing, cell cycle control and transcriptional regulation *BRCA1* may contribute to its tumor suppressor activity [10, 11].

BRCA1 and BRCA2 are also human tumor suppressor genes [6, 12], designed to chromosome 17q21 which encodes a nuclear protein of 1863 amino acids [9] that regulate transcriptional activation, DNA repair, apoptosis, cell-cycle checkpoint control, and chromosomal remodeling [13]. On the other hand, *BRCA2* located to chromosome 13q12-q13 [14] and coding for a protein of 3418 amino acids [15, 16]. Of the breast cancer susceptibility genes which have been identified nowadays, BRCA1 and BRCA2 are the most fundamental "high-risk" genes with several cases of breast and ovarian cancer accounting for most of the families [17]. If *BRCA1* or *BRCA2* is damaged by a BRCA mutation, damaged DNA is not repaired properly, and this increases the risk for breast cancer [18, 19]. P-glycoprotein 1 (P-gp1), encoded by multidrug resistance protein 1 (MDR1), is an important protein of the cell membrane that pumps many foreign substances out of cells.

Most of the initially responsive breast tumors acquire a multidrug resistance phenotype [20, 21]. The development of a multidrug-resistant phenotype in metastatic breast cancer is primarily responsible for the failure of current treatment regimens [22, 23]. Resistance to multiple drugs (MDR) is defined as efflux activity, which may decrease intracellular chemotherapeutic concentrations, thus explaining the failure of treatment in human cancers [24, 25]. Consequently, P-gp overexpression is one of the main mechanisms behind decreased intracellular drug accumulation and development of MDR cancers [25, 26].

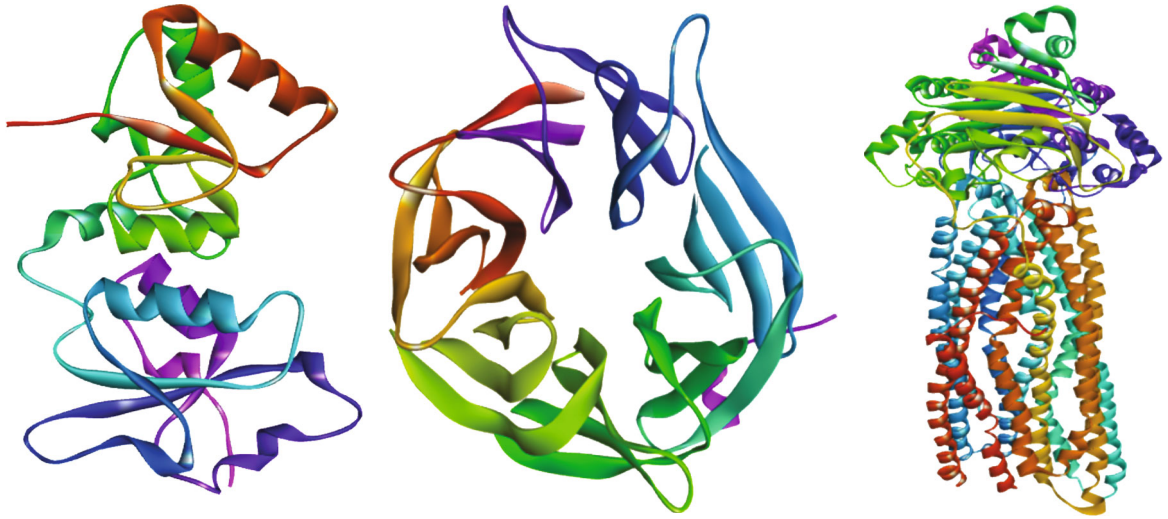
One efficient approach used to screen potential active compounds against specific target proteins, such as BRCA1 [27], BRCA2 [28] and MDR1 [29], is molecular docking simulation [30–37]. Therefore, these are important target for the design of potential anticancer activity.

2. Computational Methods

2.1. In Silico Prediction of Activity Spectra for Substances (PASS). Prediction of anticancer activity of 16 natural compounds was done with the help of computer program, PASS (prediction of activity spectra for substances). Software estimates predicted activity spectrum of a compound as probable activity (Pa) and probable inactivity (Pi) [38]. The prediction of activity is based on structure-activity relationship analysis of the training set containing more than 200,000 compounds exhibiting more than 3800 kinds of biological activities. The values of Pa and Pi vary between 0.000 and 1.000. Only activities with $Pa > Pi$ are considered as possible for a particular compound. If $Pa > 0.7$, the probability of experimental pharmacological action is high and if $0.5 < Pa < 0.7$, probability of experimental pharmacological action is less. If the value of $Pa < 0.5$, the chance of finding the activity experimentally is less, but it may indicate a chance of finding a new compound [39–41].

2.2. Ligand Preparation. The main phytochemicals and one approved drug for breast cancer treatment were downloaded from PubChem in the SDF file format. PubChem is a database for chemical molecules [42] (Figure 1).

The system is maintained by the National Centre for Biotechnology Information (NCBI), a component of the National Library of Medicine. By using Gaussian view 09 and Chem3D Pro12.0 program packages [43], all internal energies of the ligands were optimized.



(a) (b) (c)

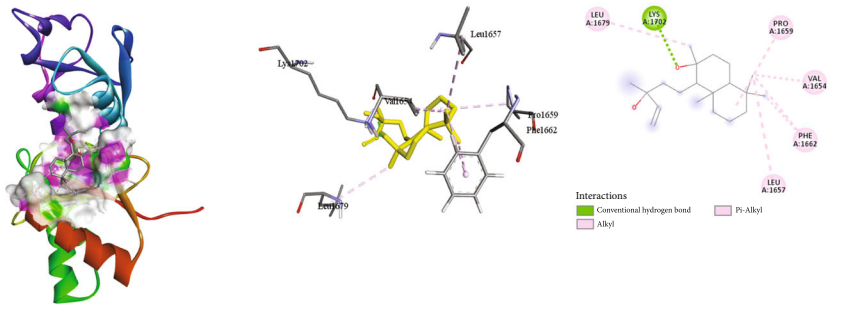
Figure 2: The three-dimensional structure of (a) BRCA1 (4Y2G), (b) BRCA2 (3EU7), and (c) MDR1 (6C0V).

Table 1: Docking results for some natural compounds against BRCA1, BRCA2, and MDR1 proteins.

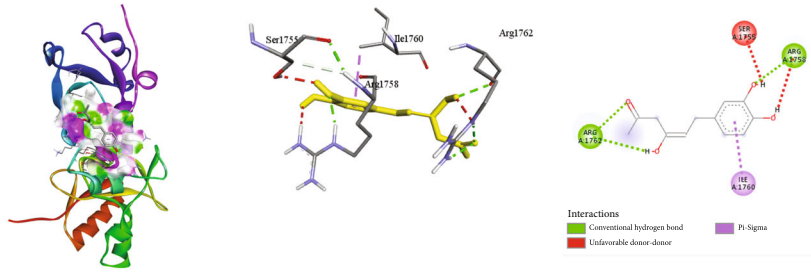
Compounds	BRCA1 (4Y2G)	BRCA2 (3EU7)	MDR1 (6C0V)
α-Hederin	-6.9	-11.0	-8.3
Andrographolide	-6.2	-8.2	-9.2
Apigenin	-6.2	-7.7	-9.0
Ascorbic acid	-5.1	-5.8	-6.3
Asiatic acid	-6.5	-8.9	-8.1
Auricularic acid	-6.8	-7.8	-8.5
Citrinin	-7.5	-7.6	-7.6
Curcumin	-7.2	-6.7	-8.3
Hispolon	-7.8	-6.7	-7.4
Nerol	-4.8	-5.8	-5.6
Phytol	-4.9	-6.4	-6.0
Retinol palmitate	-5.8	-6.1	-6.2
Sclareol	-9.8	-7.2	-6.8
Sinularin	-6.5	-9.0	-8.4
Thymol	-5.2	-6.5	-6.7
Thymoquinone	-5.2	-6.4	-7.4
5-Fluorouracil	-4.6	-5.0	-5.2

Table 2: Docking results for the best binding affinity with BRCA1 (4Y2G) protein for ten compounds.

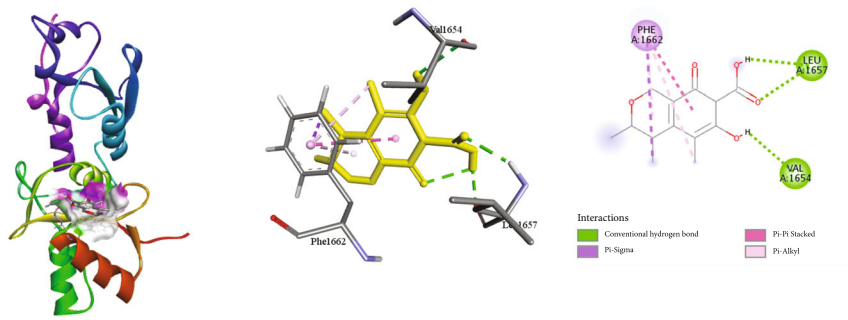
Drug	Binding affinity (kcal/mol)	No. of H-bond	Residues
Sclareol	-9.8	1	Lys1702, Leu1657, Leu1679, Pre1662, Pro1659, Val1654
Hispolon	-7.8	2	Arg1758, Arg1762, Ile1760, Ser1755
Citrinin	-7.5	2	Val1654, leu1657, Phe1662
Curcumin	-7.2	1	leu1657, Phe1662
α-Hederin	-6.9	2	VAL1810, GLU1836, PRO1812, ILE1855, PHE1821, HIS1822
Andrographolide	-6.2	2	PRO1659, LEU1657, PHE1662
Apigenin	-5.8	1	LEU1657, PHE1662, VAL1654, PRO1659
Auricularic acid	-6.2	0	PHE1662, VAL1654, PRO1659
Sinularin	-6.8	1	LEU1657, PHE1662
Asiatic acid	-6.5	2	Leu1657, Lys1702, Phe1662
5-Fluorouracil	-4.5	4	Gly1656, Leu1657, Lys1702, Val1654, Phe1662



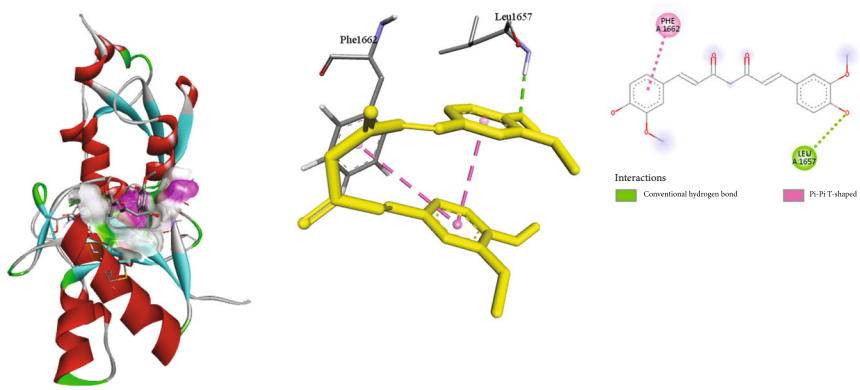
Sclareol-BRCA1



Hispolon-BRCA1



Citrinin-BRCA1



Curcumin-BRCA1

Figure 3: The four best docking results for the best screened compounds with BRCA1 (PDB: 4Y2G) protein.

Table 3: The ten best docking results for the best binding affinity with BRCA2 (3EU7) protein.

Drug	Binding affinity (kcal/mol)	H-bond	Residues
α-Hederin	-11.0	6	Val925, Val928, His1061, Pro926, Gly1166, Ala874, Leu970, Lys1062, Pro924
Andrographolide	-8.2	4	Gln1020, Gly1021, Leu1143, Leu1142, Phe1071, Leu1092, Tyr1064
Asiatic acid	-8.9	2	Gly1166, Val1123, Pro924, Pro926
Sinularin	-9.0	0	Leu1143, Val1073, Leu1092, Leu1142, Tyr1064, Phe1071
Auricularic acid	-7.8	0	Leu1092, Leu1143, His1126, Phe1071, Tyr1064
Apigenin	-7.7	3	Asp927, Val928, Trp1164,Lys1124, Pro824, Pro926
Citrinin	-7.6	2	Gln1020, Glu1066, Leu1092, Leu1142, leu1143, Phe1071, Tyr1064
Sclareol	-7.2	1	Leu1142, Leu1092, leu1143, Phe1071, Tyr1064
Curcumin	-6.7	1	Glu1066, leu1143, Phe1071, Tyr1064
Hispolon	-6.7	3	Gly1166, Val925, Val928, Met875
5-Fluorouracil	-5.0	3	Val925, Val928, Asp927

2.3. Protein Collection. The crystal structures of proteins including BRCA1 (4Y2G) [27], BRCA2 (3EU7) [28], and MDR1 (6C0V) [29] (Figure 2) were collected from the Protein Data Bank (PDB) database [44]. For the purpose of energy minimization crystal structure, we utilized Swiss-PDB Viewer software package (version 4.1.0), and then, all the heteroatoms and water molecules of proteins are removed by using PyMOL (version 1.7.4.5) before docking [45]. Both the proteins and drug structures, for the analysis of docking results, are taken into PDBQT format finally [46].

2.4. Docking Analysis and Determination of Binding Site. In *in silico* study, molecular docking is a brilliant instrument which is used for predicting the drugs candidate's pharmacodynamics profile by scoring and orienting them to the receptor binding sites [47]. Docking result determines the measure of ligand interaction to the active site of the targeted protein. The docking outcome specifies the degree of ligand interaction with the desired protein's active site. The active binding sites of the target protein are the locations of the ligand in the initial target protein grids ($40 \times 40 \times 40$), with PyMOL, AutoDock Vina [48, 49], and the active sites are the coordinates of the target protein ligand, and these active target protein binding sites were analyzed using Drug Discovery Studio version v.20.1.0.19295 and 3D Ligand Site Virtual Tool [50, 51].

3. Results and Discussion

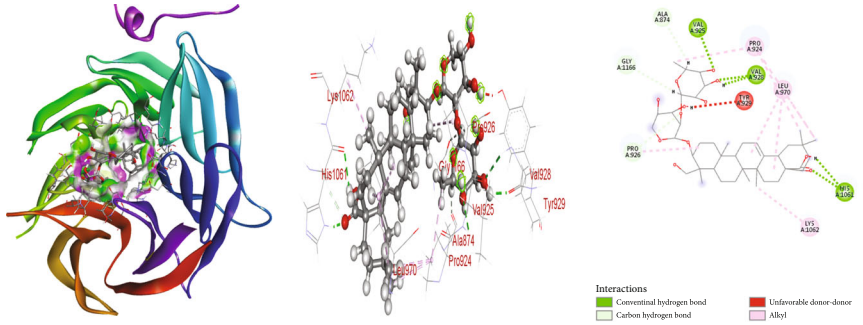
3.1. Binding Energy of the Ligand-Protein Complex. The docking is essential to predict the stronger binder and virtually screen a database of compounds. The compounds α-hederin, andrographolide, apigenin, ascorbic acid, asiatic acid, auricularic acid, citrinin, curcumin, hispolon, nerol, phytol, retinol palmitate, sclareol, sinularin, thymol, thymoquinone, and 5-fluorouracil displayed binding energy including -6.9, -6.2, -6.2, -5.1, -6.5, -6.8, -7.5, -7.2, -7.8, -4.8, -4.9, -5.8, -9.8, -6.5, -5.2, -5.2, and -4.6 (kcal/mol), respectively, against BRCA1 (4Y2G). Here, asiatic acid had the highest binding energy compared to the other compounds.

On the contrary, α-hederin, andrographolide, apigenin, ascorbic acid, asiatic acid, auricularic acid, citrinin, curcumin, hispolon, nerol, phytol, retinol palmitate, sclareol, sinularin, thymol, thymoquinone, and 5-fluorouracil, against BRCA2 (3EU7), exhibited binding energy including -11.0, -8.2, -7.7, -5.8, -8.9, -7.8, -7.6, -6.7, -6.7, -5.8, -6.4, -6.1, -7.2, -9.0, -6.5, -6.4, and -5.0 (kcal/mol). In this case, α-hederin produced the highest binding energy compared to the other compounds.

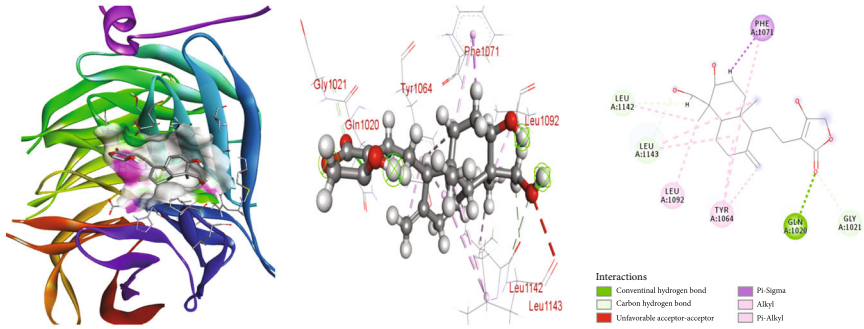
Moreover, α-hederin, andrographolide, apigenin, ascorbic acid, asiatic acid, auricularic acid, citrinin, curcumin, hispolon, nerol, phytol, retinol palmitate, sclareol, sinularin, thymol, thymoquinone, and 5-fluorouracil showed binding energy including -8.3, -9.2, -9.0, -6.3, -8.1, -8.5, -7.6, -8.3, -7.4, -5.6, -6.0, -6.2, -6.8, -8.4, -6.7, -7.4, and -5.2, respectively, against MDR1 (6C0V) (Table 1). The compound andrographolide displayed the highest binding energy compared to the other compounds.

3.2. Interaction and Binding Affinity of Compounds towards BRCA1 (4Y2G) Protein. Here, the ten best natural compounds such as sclareol, hispolon, citrinin, curcumin, α-hederin, andrographolide, apigenin, auricularic acid, and sinularin exhibited the binding affinities -9.8, -7.8, -7.5, -7.2, -6.9, -6.2, -5.8, -6.2, -6.8, and -6.5 kcal/mol (Table 2), respectively. Sclareol generated higher binding energy compared to the other derivatives against BRCA1 (4Y2G). The noncovalent interactions were calculated using Discovery Studio Software demonstrated that all the compounds exhibited both hydrogen and hydrophobic bonds that not only promoted the binding affinity but also improved binding specificity.

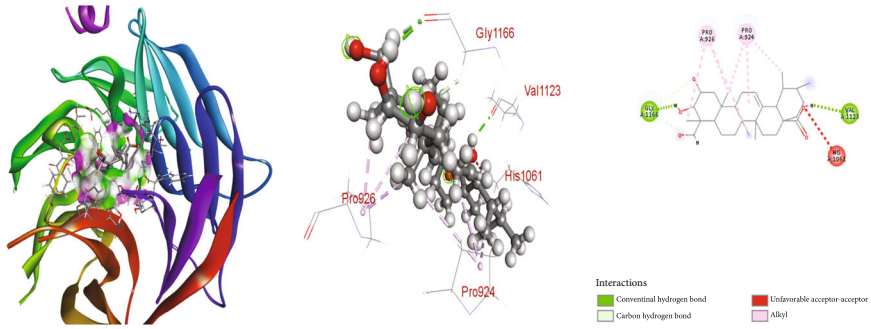
The compound, sclareol, showed strong hydrogen bonding with Lys1702 and hydrophobic interactions with Leu1657, Leu1679, Pre1662, Pro1659, and Val1654



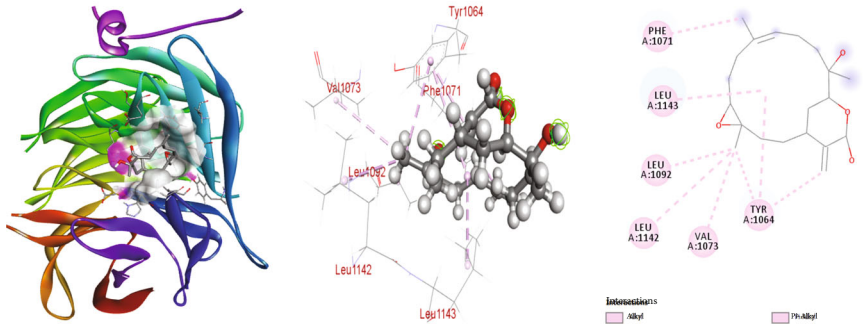
α-Hederin-BRCA2



Andrographolide-BRCA2



Asiatic acid-BRCA2



Sinularin-BRCA2

Figure 4: Docking results for the four best screened compounds with BRCA2 (3EU7) protein.

Table 4: The ten best docking results for the best binding affinity with MDR1 (6C0V) protein.

Drug	Binding affinity (kcal/mol)	H-bond	Residues
α-Hederin	-8.3	2	LYS48, MET51, ALA233, ILE352, ILE190, PHE37, TRP136, PHE193, PHE194, PHE355
Andrographolide	-9.2	4	SER434, THR906, GLN475, LEU1176, ASN903, ARG905
Apigenin	-9.0	1	TYR310, PHE728, PHE732, LEU339, PHE335, PHE759, LEU339
Auricularic acid	-8.5	1	LYS234, ALA233, ILE352, ILE190, PHE37, PHE193, PHE194, PHE355
Asiatic acid	-8.1	3	GLU564, SER1071, VAL1052, PHE512
Sinularin	-8.4	3	GLN1118, GLU1119, SER1117, ALA529, LYS536
Curcumin	-8.3	3	Arg905, Asp1171, Lys1172, Val168
Citrinin	-7.6	2	Asp164, Arg905, Asp1124, Val168
Hispolon	-7.4	2	His166, Phe904, Phe163, Thr1174
Thymoquinone	-7.4	1	Phe904, PHE194, PHE355, Asp1124, Val168
5-Fluorouracil	-5.2	4	Arg905, Glu902, Ile901, Phe904, Phe163, Val168

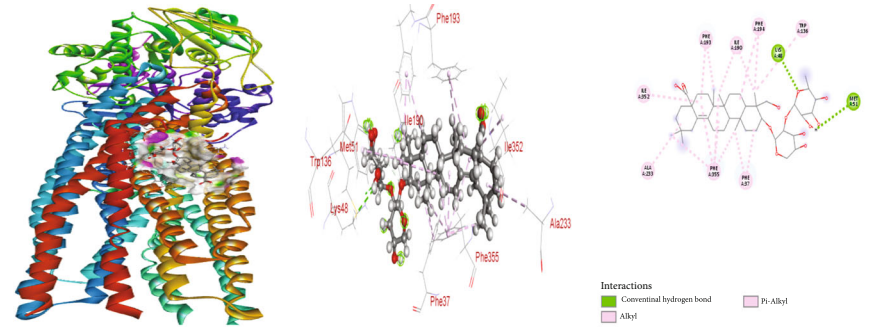
residues but hispolon and citrinin exhibited only one strong H-bond with Arg1758, Arg1762, Val1654, and leu1657, respectively, as well as hydrophobic interactions with Ile1760 and Ser1755 for hispolon and with Phe1662 for citrinin. Curcumin exhibited one hydrogen bond with leu1657 and one hydrophobic bond with Phe1662, whereas 5FU exerted four H-bonds (Gly1656, Leu1657, Lys1702, and Val1654) and other hydrophobic interactions (Phe1662) with BRCA1. The other compounds have several hydrophobic interactions with BRCA1 protein residues (Table 2). The 2D and 3D structures of nonbond interactions are given in Figure 3.

3.3. Interaction and Binding Affinity of Compounds towards BRCA2 (3EU7) Protein. The binding affinities of α-hederin, andrographolide, asiatic acid, sinularin, auricularic acid, apigenin, citrinin, sclareol, curcumin, and hispolon are -11.0, -8.2, -8.9, -9.0, -7.8, -7.7, -7.6, -7.2, -6.7, and -6.7 kcal/mol (Table 3), respectively. In comparison to α-hederin, the other compounds showed lower binding results and α-hederin exhibited -11.0 kcal/mol against BRCA2 (3EU7). The nonbond interactions using the Discovery Studio Software suggested that these compounds with BRCA2 have both hydrogen and hydrophobic interactions (Figure 4) that successfully augmented the binding interactions. We found six strong hydrogen bonds (Table 3) including carbon and conventional H-bonds with amino residues including Val925, Val928, His1061, Pro926, Gly1166, and Ala874 in the α-hederin compound; four hydrogen bonds with Gln1020, Gly1021, Leu1143, and Leu1142 in andrographolide; and two hydrogen bonds with GLY1166 and VAL1123 in asiatic acid. In α-hederin, several hydrophobic interactions such as alkyl bonds were observed with Leu970, Lys1062, and Pro924.

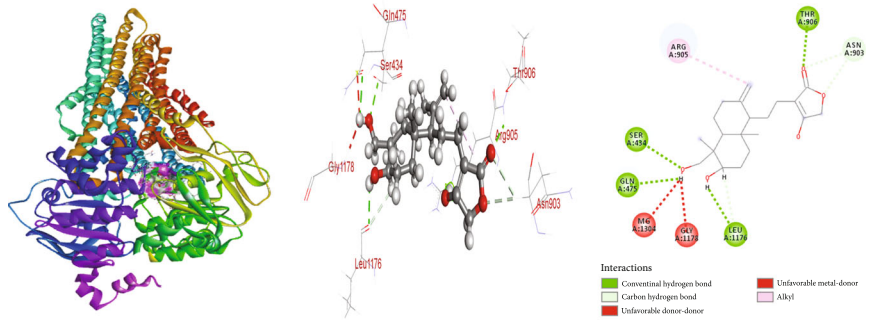
On the other hand, andrographolide exhibited one pi-sigma bond with Phe1071 as well as alkyl and pi-alkyl with Leu1092 and Tyr1064 residues. Besides, asiatic acid generated two alkyl bonds with Pro924 and Pro926. Sinularin had several hydrophobic interactions with Leu1143, Val1073, Leu1092, Leu1142, Tyr1064, and Phe1071 residues (Figure 4). The 2D and 3D structures of nonbond interactions are given in Figure 4.

3.4. Interaction and Binding Affinity of Compounds towards MDR1 (6C0V) Protein. The binding affinities of these six compounds including α-hederin, andrographolide, apigenin, asiatic acid, auricularic acid, and sinularin, curcumin, citrinin, hispolon, thymoquinone are -8.3, -9.2, -9.0, -8.5, -8.1, -8.4, -8.3, -7.6, -7.4, and -7.4 kcal/mol, respectively (Table 4). Among those compounds, the andrographolide exhibited significantly improved binding energy compared to the other compounds binding with MDR1 protein. Herein, the nonbond interactions were performed by utilizing the Discovery Studio Software and we found that both drugs had efficient interactions with amino acid residues and both drugs showed strong hydrogen bonding (Table 4) with amino residues (Figure 5) including α-hederin with Lys48 and Met51; andrographolide with Ser434, Gln475, and Leu1176; apigenin with Tyr310; and auricularic acid with Lys234.

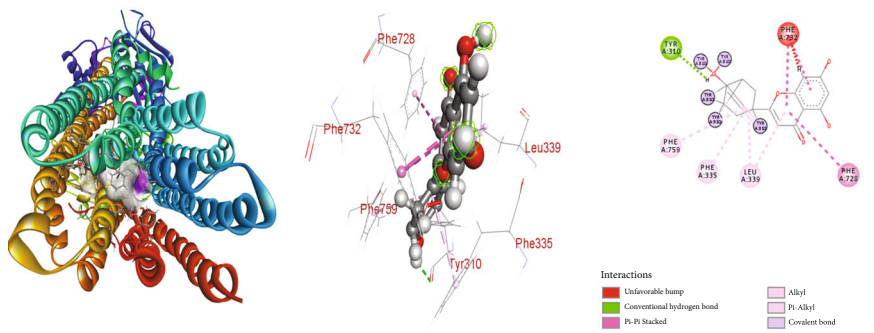
These hydrogen bonds promoted the nonbond interactions. Beyond the H-bond interactions, hydrophobic interactions play a crucial function in nonbond interaction and in this study, we observed several hydrophobic interactions (Table 4) and the residues including ALA233, ILE352, ILE190, PHE37, TRP136, PHE193, PHE194, and PHE355 with α- hederin; Thr906, Asn903, and Arg905 with andrographolide; PHE728, PHE732, LEU339, PHE335, PHE759, and LEU339 with apigenin; Ala233, Ile352, Ile190, Phe37, Phe193, Phe194; and Phe355 with auricularic acid. The 2D and 3D structures of nonbond interactions are given in Figure 5.



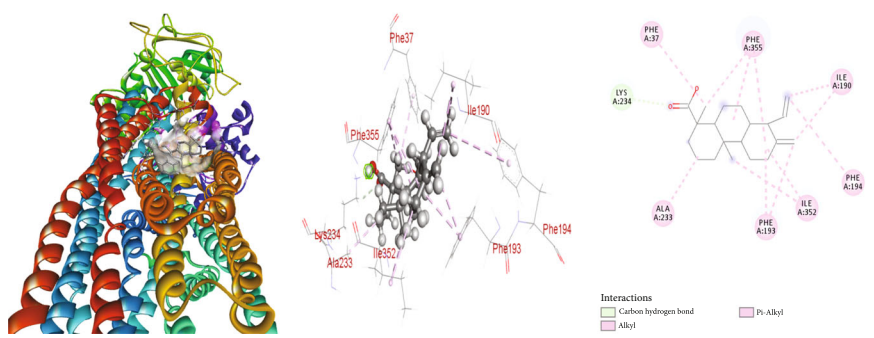
α-Hederin-MDR1



Andrographolide-MDR1



Apigenin-MDR1



Auricularic acid-MDR1

Figure 5: The four best docking results for screened compounds with MDR1 (6C0V) protein.

4. Conclusion

It can be concluded from the overall study that α-hederin, andrographolide, apigenin, asiatic acid, auricularic acid, and sinularin have potent inhibitory activity against cancer proteins (BRCA1, BRCA2, and MDR1) compared to the other compounds. All the compounds exhibited significant binding energies and the noncovalent bonds compared to the other compounds. Nevertheless, α-hederin, andrographolide, apigenin, asiatic acid, auricularic acid, and sinularin successfully docked with BRCA1, BRCA2, and MDR1 proteins as these compounds have the activity for inhibiting cancer. The nonbonding interactions can effectively target the proteins for the inhibition of cancer. We have claimed from the overall studies that α-hederin, andrographolide, apigenin, asiatic acid, auricularic acid, and sinularin will be the best conformer for BRCA1, BRCA2, and MDR1-conduced cancer for the future researchers. Our findings, in this way, could be manifested in clinical practice.

Abbreviations

BRCA1: Breast tumor suppressor gene 1
BRCA2: Breast tumor suppressor gene 2
MDR: Multiple drug resistance
MDR1: Multidrug resistance protein 1
PASS: Prediction of activity spectra for substances
P-gp: P-glycoprotein.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

SH003 and Docetaxel Show Synergistic Anticancer Effects by Inhibiting EGFR Activation in Triple-Negative Breast Cancer

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Although many anticancer drugs have been developed for triple-negative breast cancer (TNBC) treatment, there are no obvious therapies. Moreover, the combination of epidermal growth factor receptor- (EGFR-) targeted therapeutics and classical chemotherapeutic drugs has been assessed in clinical trials for TNBC treatment, but those are not yet approved. Our serial studies for newly developed herbal medicine named SH003 provide evidence of its broad effectiveness in various cancers, especially on TNBC. The current study demonstrates a synergic effect of combinatorial treatment of SH003 and docetaxel (DTX) by targeting EGFR activation. The combinatorial treatment reduced the viability of both BT-20 and MDA-MB-231 TNBC cells, displaying the synergism. The combination of SH003 and DTX also caused the synergistic effect on apoptosis. Mechanistically, the cotreatment of SH003 and DTX inhibited phosphorylation of EGFR and AKT in both BT-20 and MDA-MB-231 cells. Moreover, our xenograft mouse tumor growth assays showed the inhibitory effect of the combinatorial treatment with no effect on body weight. Our immunohistochemistry confirmed its inhibition of EGFR phosphorylation in vivo. Collectively, combinatorial treatment of SH003 and DTX has a synergistic anticancer effect at a relatively low concentration by targeting EGFR in TNBC, indicating safety and efficacy of SH003 as adjuvant combination therapy with docetaxel. Thus, it is worth testing the combinatorial effect in clinics for treating TNBC.

1. Introduction

Breast cancer is the leading cause of cancer-related death among women worldwide and has first surpassed lung cancer, with an estimated 2,261,419 new cases and 684,996 deaths in 2020 [1]. Breast cancer is commonly classified into three subtypes based on the expression status of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Among these subtypes, triple-negative breast cancer (TNBC) is characterized by an absence of ER and PR and a lack of HER2 overexpression. Although other breast cancer subtypes including ER+/PR+/HER2-, ER+/PR-/HER2-, and HER2 overexpression have been managed by targeted therapy such as endocrine therapy and anti-HER2 therapy with improved prognosis, there are no effective therapeutic ways for TNBC [2–4].

Epidermal growth factor receptor (EGFR) suffers autophosphorylation at multiple tyrosine residues via its interaction with its endogenous ligand and activates downstream signaling pathways including ERK, AKT, and STAT3 [5].

Therefore, EGFR-mediated events are multifaceted in the cells. EGFR is overexpressed in TNBC, thereby being proposed as a therapeutic target [6]. While BRCA mutation appears to be linked to EGFR overexpression in TNBC, underlying mechanisms are still unclear [7, 8]. Moreover, gain-of-function mutations of EGFR are not broadly detected in TNBC patients [9]. EGFR-targeting agents such as gefitinib, erlotinib, osimertinib, and cetuximab are often used in clinics. Although EGFR-targeting agents are currently approved for nonsmall cell lung cancer (NSCLC) on the basis of their effectiveness, the response is limited to patients with gain-of-function mutations of EGFR such as exon 19 deletion and L858R mutation [10]. Those agents are not working in NSCLC with wild-type EGFR overexpression, which is a reason that those agents fail in TNBC treatment even though molecular and cellular mechanisms are completely unclear [11–13]. Nevertheless, clinical trials evaluating the effect of EGFR-targeting agents in combination therapies with chemotherapies are still conducted for TNBC treatment [14].

Docetaxel (DTX), a semisynthetic analog of paclitaxel isolated from European yew (*Taxus baccata*), is one of chemotherapeutics that causes cell cycle arrest by interfering with microtubules functions [15, 16]. DTX was first approved for the treatment of cancer such as breast, ovarian, and nonsmall cell lung cancer, and especially used in treatment of TNBC or early stage of human breast cancers [17]. Even though DTX is the effective anticancer drug, most chemotherapeutic agents including DTX have faced to limitations such as drug resistance, cancer recurrence, and adverse effects [18, 19]. Therefore, it is urgent to enhance the effectiveness of DTX in TNBC treatment with no adverse effect. One of therapeutic ways is likely to reduce its dose, which could ameliorate adverse effects with keeping its effectiveness.

SH003 is an herbal mixture consisting three herbs *Astragalus membranaceus* (Am), *Angelica gigas* (Ag), and *Trichosanthes Kirilowii Maximowicz* (Tk), which has been developed as a novel anticancer drug against several cancers including prostate, cervical, pancreatic, and breast cancer [20–22]. Especially, SH003 inhibits the growth of MDA-MB-231 TNBC cell line by inducing apoptosis followed by autophagy, both *in vitro* and *in vivo* [23]. Moreover, a combination of SH003 and doxorubicin exhibits synergistic effect on TNBC [24]. In addition, SH003 sensitizes paclitaxel-resistant ER-positive MCF7 cells to paclitaxel by inhibiting p-glycoprotein (MDR1) activity [25]. Based on the chemotherapeutic effect of SH003 or DTX on TNBC, we hypothesized that SH003 treatment combined with DTX may be effective for treatment of TNBC.

The present study is aimed at evaluating the synergistic effect of SH003 and DTX in TNBC cells. We showed that cotreatment of SH003 and DTX synergistically enhances apoptotic cell death in BT-20 and MDA-MB-231 cells. Both *in vitro* and *in vivo* studies demonstrated that blocking EGFR signaling is a key in the inhibitory mechanism of combination therapy. These findings suggest that combination of SH003 and DTX would be one of beneficial therapeutic strategy for TNBC treatment.

2. Materials and Methods

2.1. SH003, Chemicals, and Reagents. SH003 was prepared as described in our previous study [26]. In brief, SH003 was provided from Hanpoong Pharm and Foods Company (Jeonju, Republic of Korea). Am, Ag, and TK were mixed at 1 : 1 : 1 ratio (*w*/*w*) and then extracted with 30% ethanol at 100°C for 3 h. Dried extracts were dissolved in 30% ethanol and stored at −80°C until use. DTX (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in DMSO and stored at −20°C.

Thiazolyl blue tetrazolium bromide (MTT) powder was purchased from Sigma-Aldrich (St. Louis, MO, USA). FITC-conjugated Annexin V apoptosis Detection Kit and 7-aminoactinomycin D (7-AAD) were purchased from BD Pharmingen™ (BD Biosciences, San Jose, CA, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. Anticleaved caspase-3, -PARP, -GAPDH, -p-EGFR (Tyr 1068), -p-EGFR (Tyr 1173), -EGFR, -p-AKT (Ser 473), -AKT, -p-C-Raf (Ser 338), -p-STAT3, and -STAT3 antibodies were purchased from Cell Signaling (Danvers, MA, USA). Anti-p-ERK and ERK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Ki67 and CD31 antibodies were purchased from Abcam (Cambridge, MA, USA). Horseradish peroxidase- (HRP-) conjugated secondary antibodies for mouse and rabbit were purchased from SeraCare Life Sciences (Milford, MA, USA).

2.2. Cell Culture. Human breast cancer BT-20 and MDA-MB-231 cell lines were purchased from Korean Cell Line Bank (Seoul, Republic of Korea) and cultured in DMEM or RPMI-1640 medium (WelGENE, Daegu, Republic of Korea) supplemented with 10% fetal bovine serum (JR Scientific, Inc., Woodland, CA, USA) and 1% penicillin/streptomycin solution (WelGENE, Daegu, Republic of Korea). The cells were maintained at 37°C in 5% CO_2/95% air with 100% humidity.

2.3. MTT Assay and Combination Index Analysis. BT-20 and MDA-MB-231 cells were seeded in 96-well plates and then treated with SH003 (100, 300, and 500 μg/mL), DTX (1, 10, 100, and 1000 nM), or combination. After 24 h incubation, cell viability was measured by MTT assay with an absorbance at 570 nm. The interaction (synergistic, additive, and antagonistic effects) between SH003 and DTX was assessed by combination index (CI) analysis using Compusyn software (ComboSyn, Inc., Paramus, NJ, USA) (http://www.combosyn.com). CI value was calculated by the equation as follows [27]:

$$CI_x = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} \quad (1)$$

$(D_x)_1$ is the dose of SH003 alone that inhibits $_x$%. $(D_x)_2$ is the dose of DTX alone that inhibits $_x$%. $(D)_1$ the portion of SH003 in combination SH003 and DTX also inhibits $_x$%. $(D)_2$ is the portion of DTX in combination SH003 and DTX also inhibits $_x$%. "$CI < 1$", "$CI = 1$", and "$CI > 1$" mean synergistic, additive, and antagonistic effect, respectively.

2.4. Apoptosis Analysis by Flow Cytometry. Apoptotic cell death was analyzed by Annexin V/7-AAD double staining. Cells were stained with Annexin V followed by staining with 7-AAD in the dark for 15 min at room temperature. Stained cells were detected by FACSCalibur (BD Biosciences, San Jose, CA, USA), and apoptotic cells were analyzed using CellQuest Pro version 5.2 (BD Biosciences, San Jose, CA, USA) software.

2.5. TUNEL Assay. DNA fragmentation was measured using TUNEL assay (Abcam, Cambridge, MA, USA), according to the manufacturer's protocol. After the treatment with SH003, DTX, or combination for 24 h, the cells were harvested and fixed in 4% paraformaldehyde (PFA) for 15 min at 4°C. The cells were washed with PBS, resuspended in 100 μL of PBS, and then added 1 mL of 70% EtOH for 30 min on ice. Subsequently, the cells were washed twice in wash buffer and incubated with 50 μL of DNA labeling solution containing TdT enzyme and Br-dUTP for 1 h at 37°C. The cells were added rinse buffer and centrifuged. Then, the samples were resuspended in 50 μL of anti-BrdU-Red antibody solution for 30 min at room temperature in the dark. Stained cells were diluted with rinse buffer and analyzed in FL-2 channel by flow cytometry.

2.6. Transfection. Cells were seeded in 6-well plate and transfected with 1.5 μg pCDNA3-Myr-HA-AKT2 (addgene #9016) plasmid using lipofectamine 3000 (Invitrogen, CA, USA). Cells were incubated in 10% FBS/antibiotic-free media for 24 h and then trypsinized and seeded for additional studies.

2.7. Western Blotting. Total protein was extracted with RIPA buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% triton X-100, 2 mM EDTA, 0.1% SDS, and 1% sodium deoxycholate. Protein concentration was quantified by Bradford assay, and proteins were separated on 8-12% SDS-PAGE. Separated proteins were transferred to nitrocellulose membrane and blocked with 5% skim milk in PBS-T at room temperature for 1 h. The membrane was incubated with primary antibodies at 4°C overnight, washed, and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. Proteins were measured using the EZ-western detection kit (Dogen-Bio, Seoul, Republic of Korea).

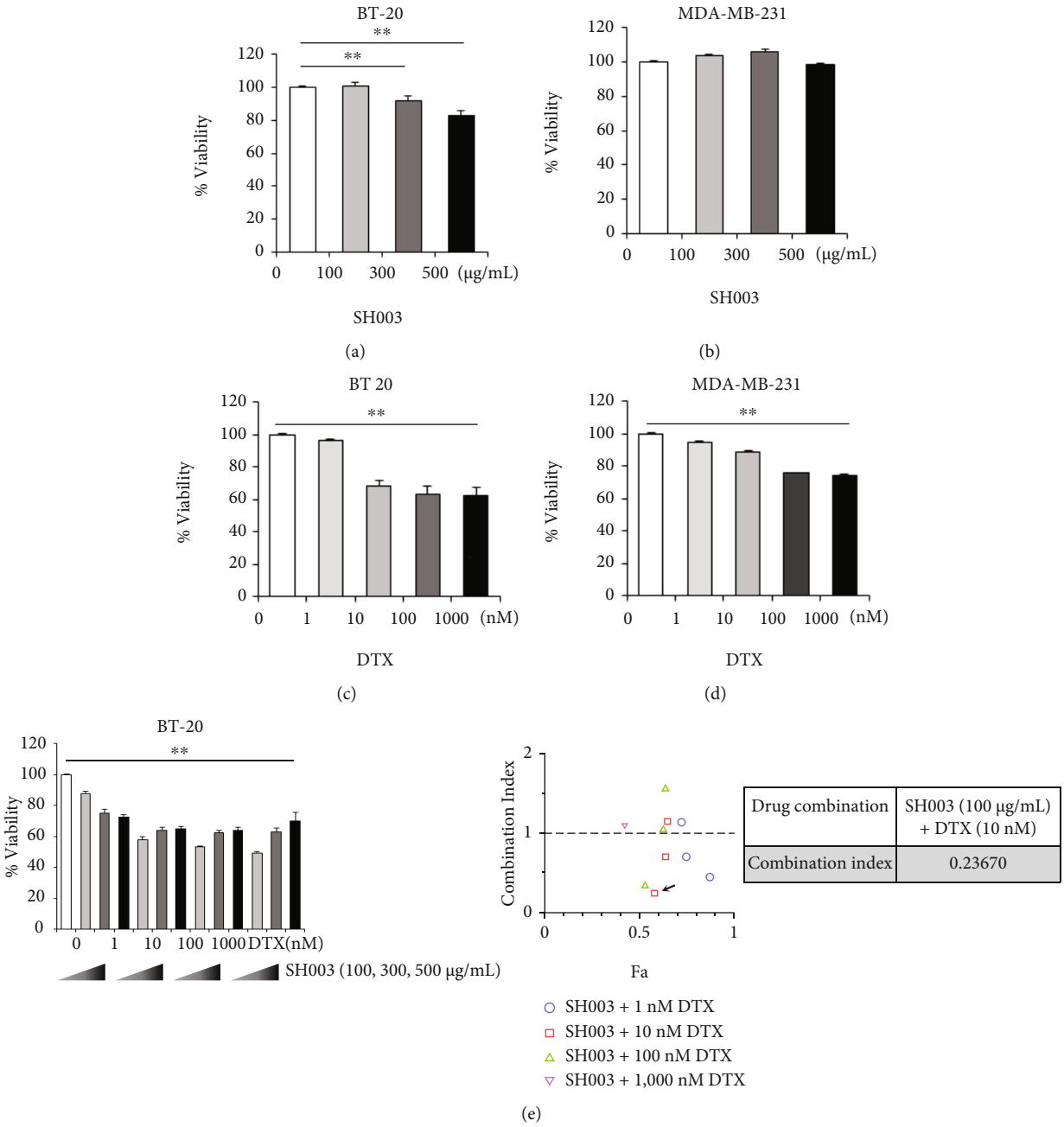
2.8. In Vivo Studies. All animal studies were approved by Kyung Hee University Institutional Animal Care and Use Committee (KHU-IACUC). Five-week-old female BALB/c nude mice were purchased from Nara Biotech (Seoul, Republic of Korea). Mice were given to access to food and drinking water *ad libitum* and were housed in appropriate isolated cage under pathogen-free condition with 12 h light/12 h dark cycle at room temperature (22-25°C). To establish tumor xenograft mouse model, BT-20 cell suspension (1×10^7 cells) in 100 μL PBS was subcutaneously inoculated in the right flank of mice. Based on no observed adverse effect of levels (NOAEL) of SH003 from phase 1 clinical study (4,800 mg/day) and maximum tolerable dose of DTX (75 mg/m^2) in cancer patients (NCT03081819) [28], animal equivalent dose was determined by following equation: Human equivalent dose (mg/kg) = Animal dose (mg/kg) $*$ $(\text{Weight}_{\text{animal}}\,[\text{kg}]/\text{Weight}_{\text{human}}\,[\text{kg}])^{(1-0.75)}$ [29]. Human and mice weights are 65 kg and 0.02 kg, respectively. Based on Kleiber's law [30], exponent for body surface area is 0.75, which account for difference in metabolic rate. Mice were divided into four groups; control ($n = 3$), SH003 ($n = 4$), DTX ($n = 4$), and SH003 + DTX ($n = 5$). When tumor volume reached at 100 mm^3, drugs were injected. DTX was administrated intravenously *via* tail vein once a week with 15.277 mg/kg, while DMSO to control or SH003 alone group. SH003 was orally treated three times a week with 557.569 mg/kg, while saline to control or DTX alone group. Body weights were measured three times a week, and tumor volumes were analyzed daily for 17 days. Tumor volume was calculated using the formula: widt h^2 × length/2. The mice were euthanized, and tumors were isolated. Tumor tissues were fixed and embedded in paraffin. For hematoxylin and eosin (H&E) staining, tissues mounted on slide glasses were incubated with H&E solution. The immunohistochemical analysis was performed using Vectastain ABC-AP staining kit according to manufacturer's instruction (Vector Laboratories, Burlingame, CA USA). The slide was stained with Ki-67, CD31, p-EGFR (Tyr 1068), and cleaved caspase-3 after performing heat-induced antigen retrieval using sodium citrate buffer (pH 6.0). Images of H&E and IHC were obtained with microscope (Carl Zeiss, Germany) at a magnification of 40×.

2.9. Statistical Analysis. Data were shown as the mean and standard deviation from at least three experiments. The statistical differences of means among the groups were analyzed by one-way ANOVA followed by Dunnett's or Bonferroni's test. P value < 0.05 means statistically significant differences.

3. Results

3.1. Synergistic Effect of SH003 and DTX on TNBC Cell Viability. We first investigated the cytotoxic effect on TNBC cells by either SH003 or DTX. BT-20 and MDA-MB-231 cells were exposed to various concentrations of either SH003 or DTX for 24 h. SH003 did not affect the viabilities of both BT-20 and MDA-MB-231 cells, as the viabilities were about 83% and 98% at 500 μg/mL dose, respectively (Figures 1(a) and 1(b)). However, DTX decreased the viabilities of both BT-20 and MDA-MB-231 cells in a dose-dependent manner (Figures 1(c) and 1(d)). Moreover, cotreatment of SH003 with DTX significantly inhibited the viability of TNBC cells, although not show a dose-dependent effect in cell survival (Figures 1(e) and 1(f)). To identify whether SH003 has the synergistic effect with DTX, the combination index (CI) value of drug pair was evaluated (Tables 1 and 2).

The highest synergistic effects in BT-20 and MDA-MB-231 cells were observed in combination of SH003 at 100 μg/mL and DTX at 10 nM (CI: 0.24) and in combination of SH003 at 100 μg/mL and DTX at 100 nM (CI: 0.1), respectively (Figures 1(e) and 1(f)). These findings indicated that SH003 and DTX have the synergistic effect at a relatively low concentration of DTX by blocking excessive toxicity accumulation.



Drug combination	SH003 (100 μg/mL) + DTX (10 nM)
Combination index	0.23670

Figure 1: Continued.

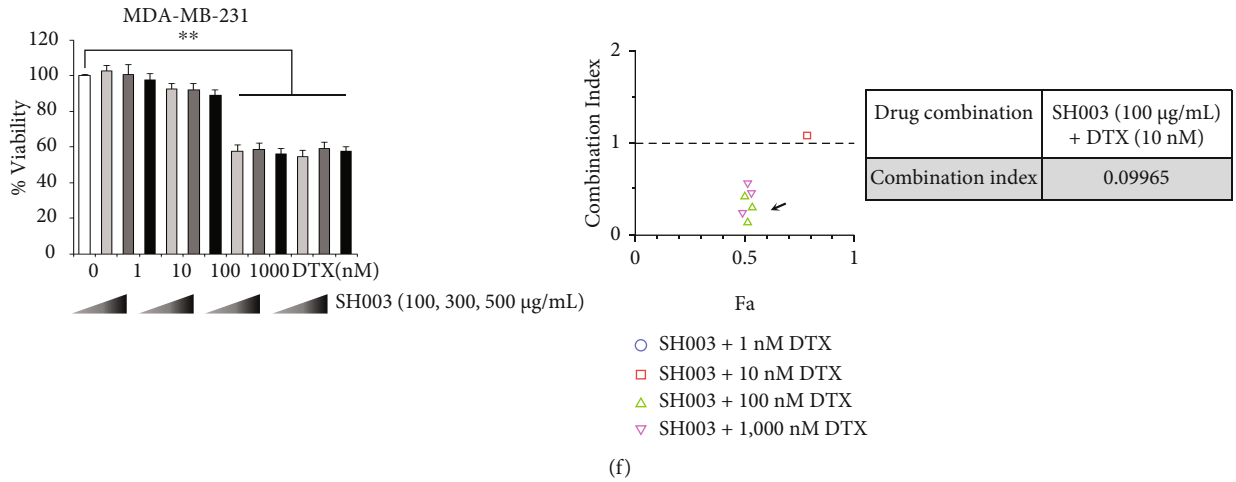


Figure 1: Combinatorial treatment of SH003 and DTX shows the synergic effect on TNBC cells. (a, b) BT-20 and MDA-MB-231 cells were treated with SH003 (100, 300, and 500 μg/mL) for 24 h. Cell viability was measured by MTT assay. (c, d) Cells were treated with DTX (1, 10, 100, and 1000 nM) for 24 h and then MTT assay was performed. (e, f) Cells were cotreated with SH003 and DTX at the indicated doses for 24 h and analyzed by MTT assay. The combination index (CI) was calculated using CompuSyn software. $CI < 1$, $CI = 1$, and $CI > 1$ indicate synergistic, additive, and antagonistic effects, respectively. Also, Fa refers to inhibitory rate. Data represent three independent experiments. One-way ANOVA was used to compare the results, $*P < 0.05$, $**P < 0.01$ vs. control.

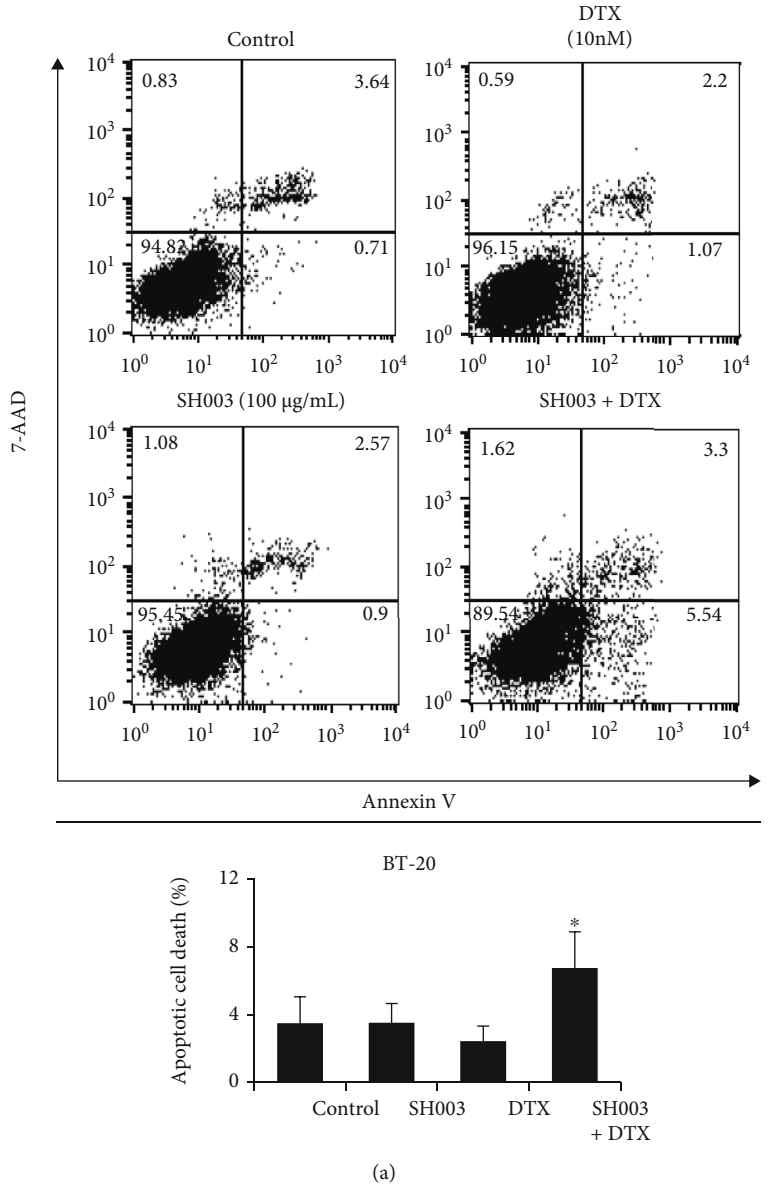
Table 1: Combination index value of SH003 and DTX in BT-20 cells.

Drug		BT-20
DTX (nM)	SH003 (μg/mL)	Combination index (CI)
	100	0.44311
1	300	0.70508
	500	1.14198
	100	0.23670
10	300	0.70086
	500	1.14927
	100	0.35019
100	300	1.05284
	500	1.57479
	100	1.12051
1000	300	4.74933
	500	10.8015

Table 2: Combination index value of SH003 and DTX in MDA-MB-231 cells.

Drug		MDA-MB-231
DTX (nM)	SH003 (μg/mL)	Combination index (CI)
	100	1.85E20
1	300	5.90E23
	500	7.01930
	100	3.82112
10	300	3.16236
	500	1.17233
	100	0.09965
100	300	0.27457
	500	0.42340
	100	0.19020
1000	300	0.45114
	500	0.57037

3.2. Combination of SH003 and DTX Induces the Apoptotic Cell Death. To examine apoptosis, BT-20 and MDA-MB-231 cells were treated with SH003 and/or DTX for 24 h, and then apoptosis was analyzed by flow cytometry. In Figures 2(a) and 2(b), the results showed that combined with SH003 and DTX induces apoptosis in both cells. In BT-20 cells, the combinatorial treatment enhanced the level of the early and late apoptotic cells, compared with SH003 or DTX alone (Figure 2(a)). Similarly, the combination of SH003 and DTX increased levels of cleaved PARP and cleaved caspase-3, which is known to marker of early apoptosis processes (Figure 2(c)). As shown in Figure 2(b), we observed that the cotreatment showed a similar increase in the early and late apoptotic cells (about 24%) with DTX (about 23%), while the increase in late apoptotic cells was higher in combinatorial treatment than in DTX. Also, apoptosis markers were induced by both DTX and cotreatment (Figure 2(d)). These results suggest that apoptosis process in MDA-MB-231 is distinct from BT-20. Therefore, we further performed the TUNEL assay to assess the induction of late apoptosis by combination treatment in MDA-MB-231 cells. The TUNEL assay can detect the DNA fragmentation, which is a representative marker occurred in end process of apoptosis [31, 32]. In BT-20 cells, SH003, DTX, and combination treatment increased the TUNEL positive cells, but there was no synergistic effect of combination treatment. On the other hand, the combination of SH003 and DTX caused an increase in TUNEL positive cells (about 10%)



(a)

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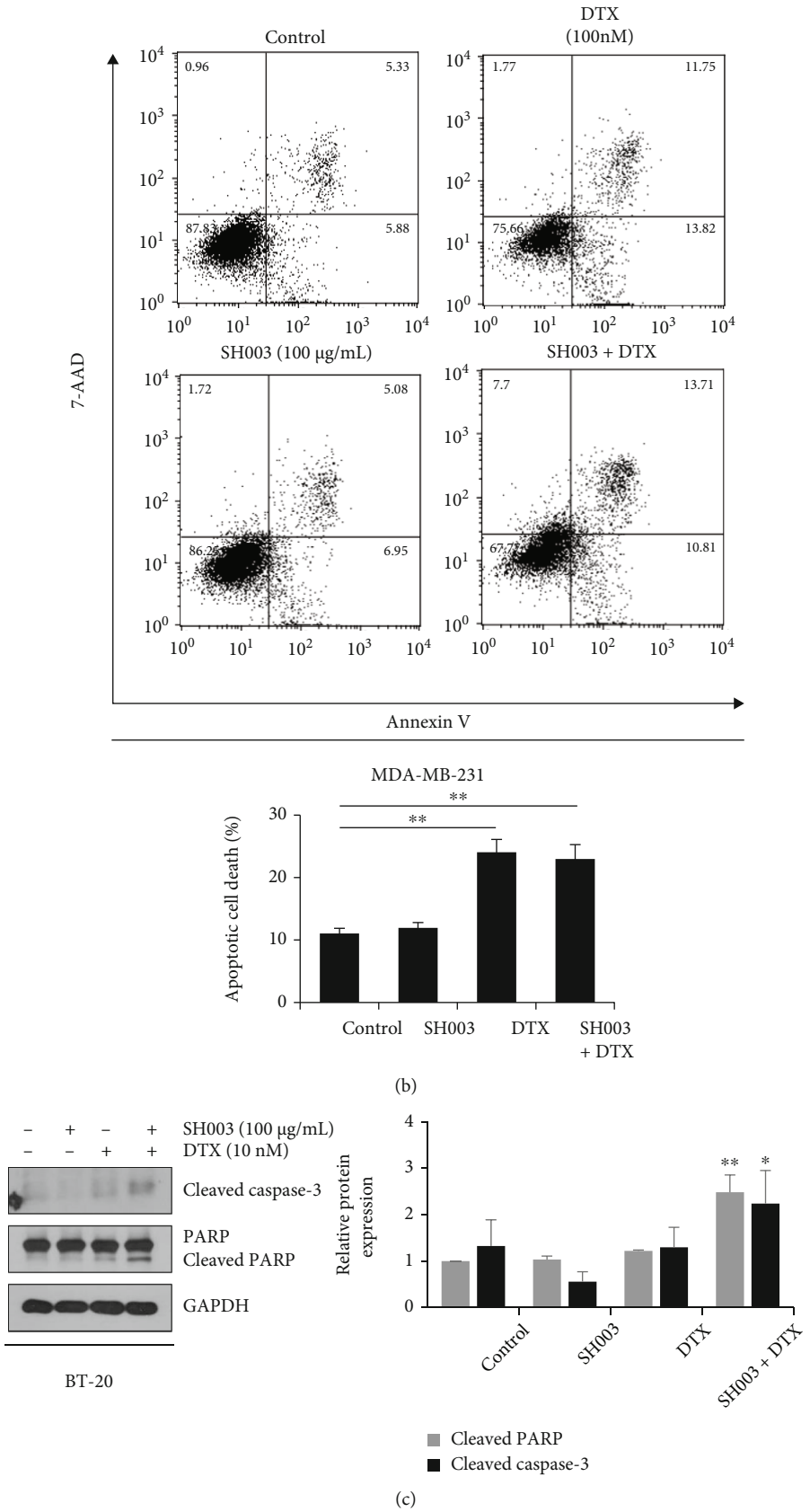

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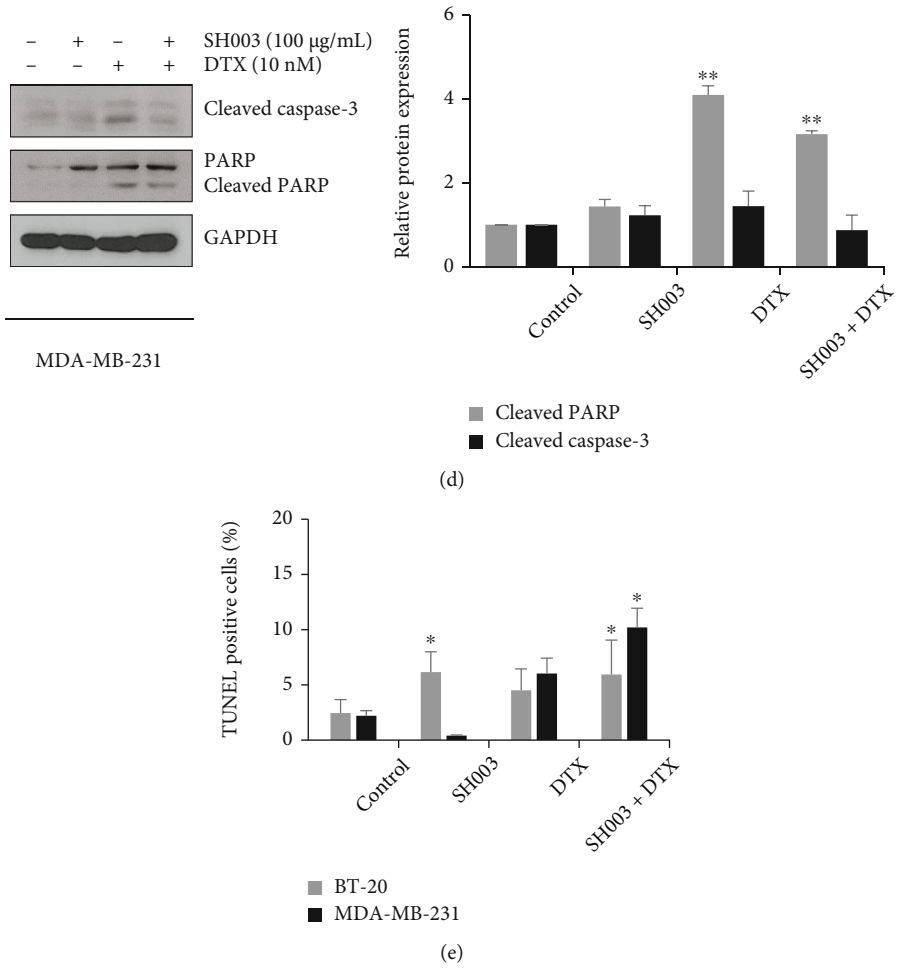
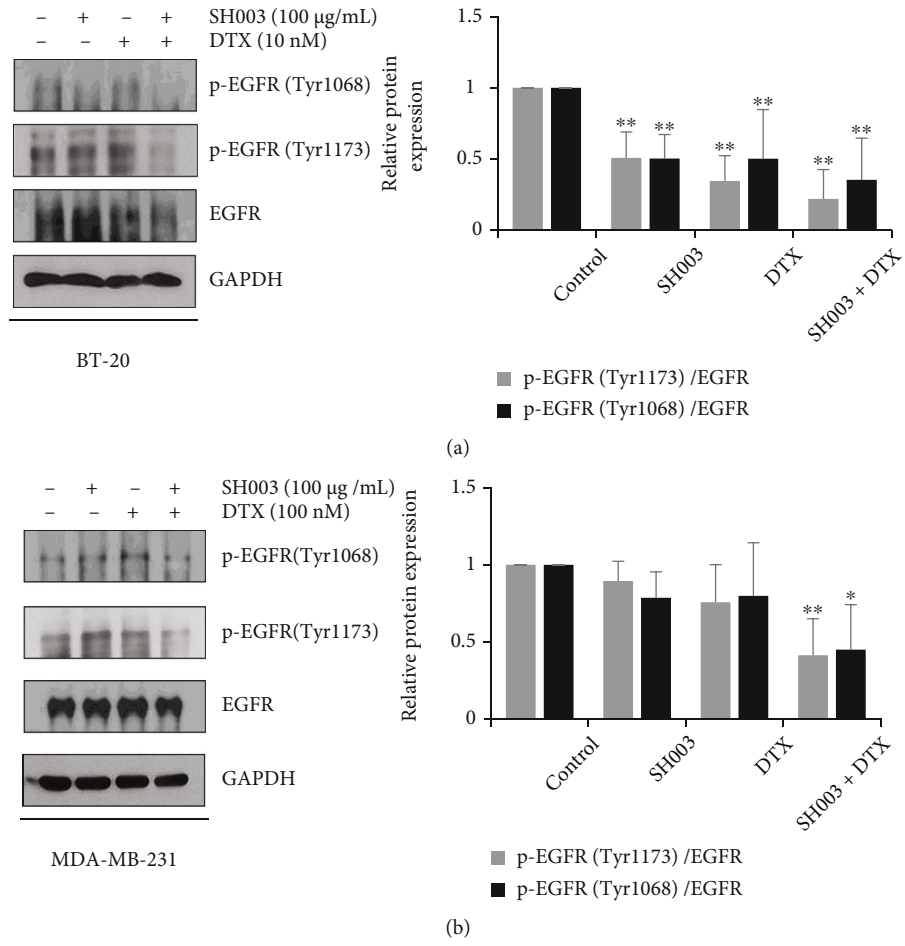


Figure 2: Combinatorial treatment of SH003 and DTX induces apoptotic cell death in TNBC cells. Cells were treated with SH003 (100 μg/mL), DTX (10 nM or 100 nM), or combination for 24 h. (a, b) Apoptosis was analyzed by flow cytometry. The bar graph indicates apoptotic cells and data were shown as the mean ± SD on three independent experiments. (c, d) Protein expression levels were determined by western blotting. The relative expression of apoptosis-related proteins was quantified using Image J software and normalized to GAPDH. Data are means ± SD on three independent experiments. (e) Cells were treated with SH003 (100 μg/mL), DTX (10 nM or 100 nM), or combination for 24 h. DNA fragmentation was detected using TUNEL assay and analyzed by flow cytometry. The bar graph shows the TUNEL positive cells. Data are expressed in the mean ± SD from three independent experiments and evaluated by one-way ANOVA, $*P < 0.05$, $**P < 0.01$ vs. control.

than DTX single (about 6%) in MDA-MB-231 cells (Figure 2(e)), indicating that combination treatment synergistically induces apoptotic cell death and especially is mediated by late apoptosis process in MDA-MB-231 cells and early/middle apoptosis in BT-20 cells. Taken together, these results demonstrated that combination treatment promotes the sensitivity of SH003 or DTX on TNBC cell by increasing the apoptosis.

3.3. SH003 Combined with DTX Induces Apoptosis through EGFR-AKT Signaling Pathway. EGFR is overexpressed in TNBC cells [6], and EGFR-targeted therapeutics have been developed for treatment [33]. Therefore, we investigated if the combination of SH003 and DTX inhibits EGFR signaling pathway. As shown in Figures 3(a) and 3(b), combinatorial treatment of SH003 and DTX inhibited phosphorylation of EGFR in both BT-20 and MDA-MB-231 cells. AKT, Raf/ERK, and STAT3 are downstream signaling factors of EGFR-mediated signaling and known to activate tumor growth and metastasis [34]. Furthermore, many studies have been focused on AKT, ERK, and STAT3 as therapeutic targets in TNBC cells [35–37]. Therefore, we assessed whether combination of SH003 and DTX regulates downstream pathway of EGFR. Compared with SH003 or DTX alone, combinatorial treatment decreased phosphorylation of AKT in both BT-20 and MDA-MB-231 cells. However, the combinatorial treatment inhibited phosphorylation of Raf/ERK and STAT3 only in BT-20 cells (Figures 3(c) and 3(d)), suggesting that molecular module of EGFR-mediated downstream signaling may be different between BT-20 and MDA-MB-231 cells.

Next, we examined whether inhibited EGFR-AKT signaling pathway is directly related to cell death mechanism by combinatorial treatment. Upon epidermal growth factor (EGF) binds to EGFR, it activates AKT-mediated cancer cell proliferation and metastasis. As shown in Figure 4(a), the



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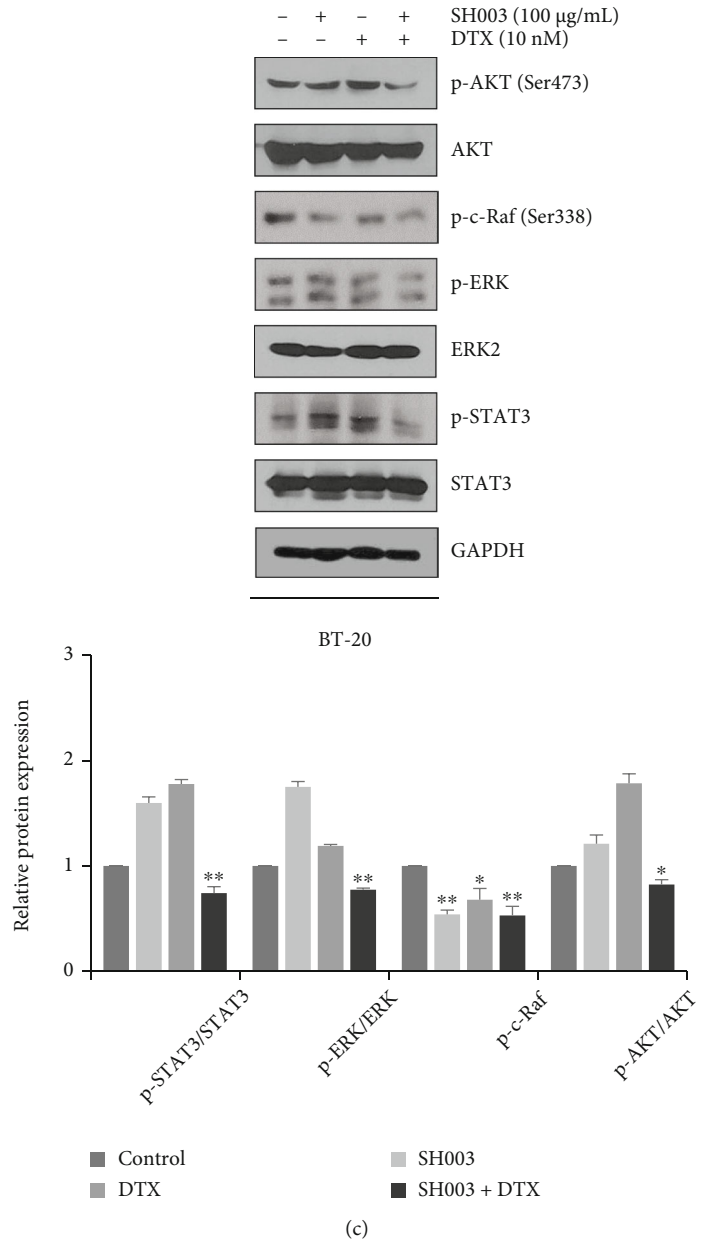

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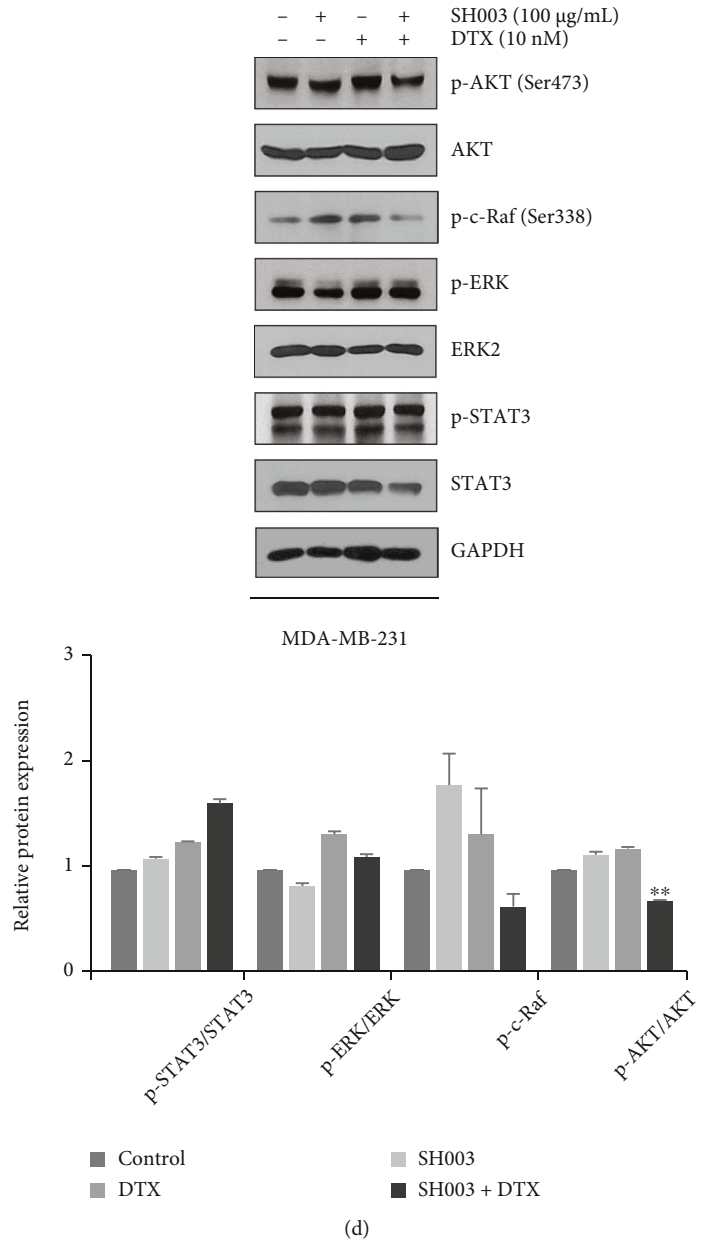
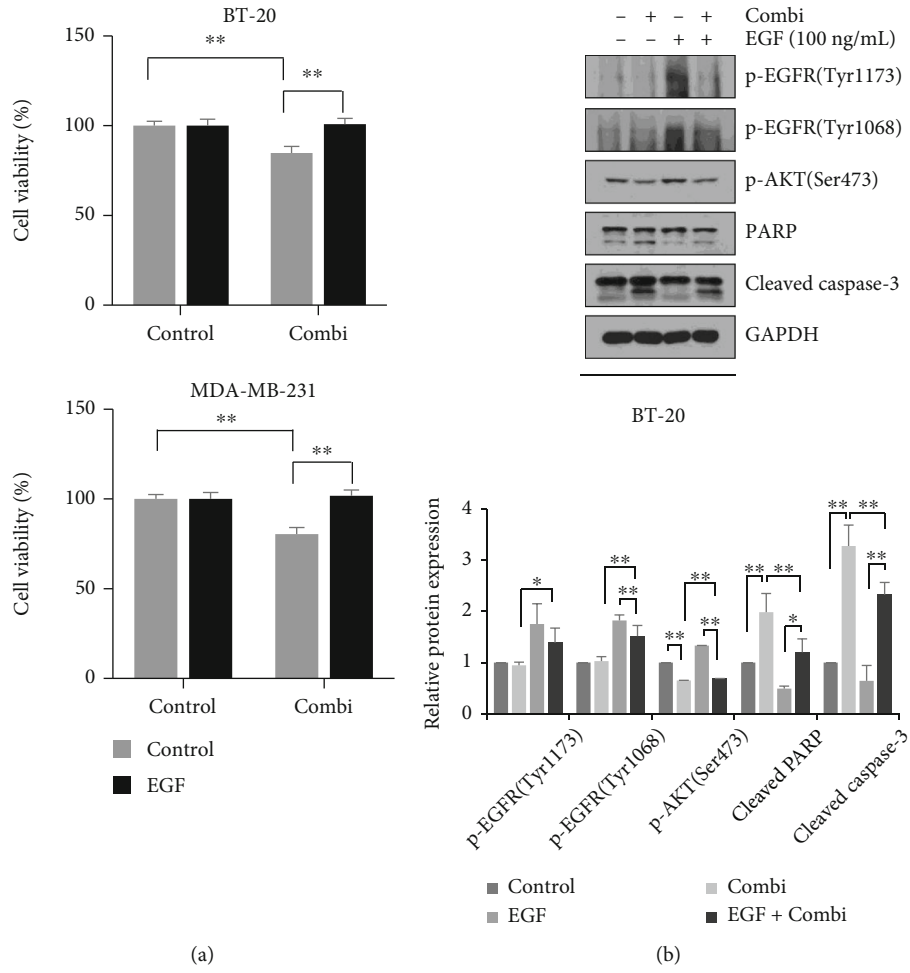


Figure 3: SH003 combined with DTX inhibits EGFR activation and EGFR downstream pathways. Cells were treated with SH003, DTX, or combination for 30 min. (a, b) Western blotting of EGFR in BT-20 and MDA-MB-231 cells. The bar graph indicates the relative protein expression and normalized to total EGFR. (c, d) Western blot analysis of EGFR downstream pathways in BT-20 and MDA-MB-231 cells. The relative expression of proteins was measured by Image J software. The data represent mean ± SD on three independent experiments. $*P < 0.05$, $**P < 0.01$ vs. control.

effect of combination was blocked by EGF treatment in two breast cancer cells. EGF treatment abolished the EGFR and AKT inactivation by combination treatment, but also increased apoptosis markers were inhibited by EGF (Figures 4(b) and 4(c)). Also, EGF rescued both BT-20 and MDA-MB-231 cells from the apoptosis induction by SH003 and DTX (Figure 4(d)), suggesting the inhibition of EGFR signaling involved in apoptotic cell death and AKT inhibition by the combined treatment. We then found the effect of downstream AKT on breast cancer cell death. In Figure 4(e), when AKT was overexpressed, the combination of SH003 and DTX increased the cell viability compared to the control group in BT-20 and MDA-MB-231 cells. In addition, AKT-overexpressed cells prevented the cleavage of PARP, an apoptotic marker, under combination treatment (Figures 4(f) and 4(g)). Based on these results, we suggest that EGFR-AKT pathway is likely to be the main targets of SH003-DTX combination treatment on breast cancer.

3.4. Combination with SH003 and DTX Suppresses the Tumor Growth and EGFR Phosphorylation In Vivo. To investigate the efficacy of SH003 combined with DTX



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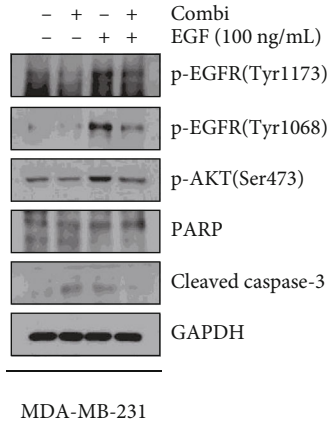

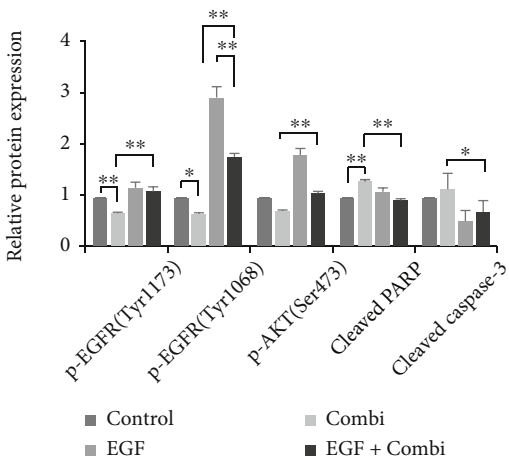

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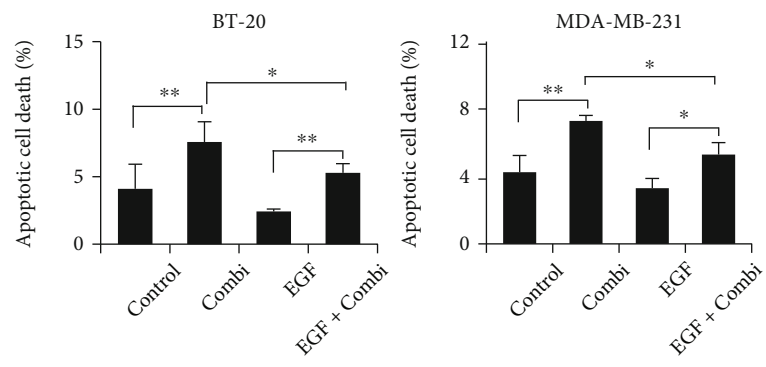

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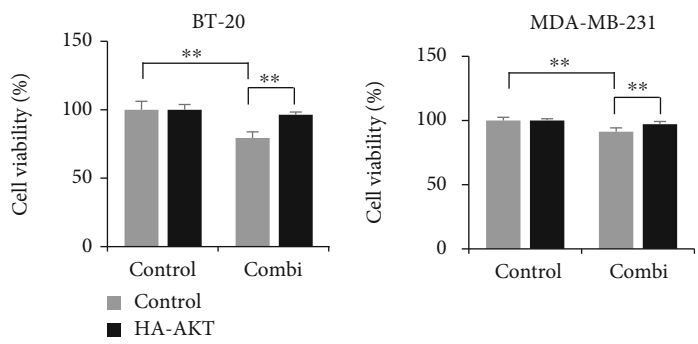

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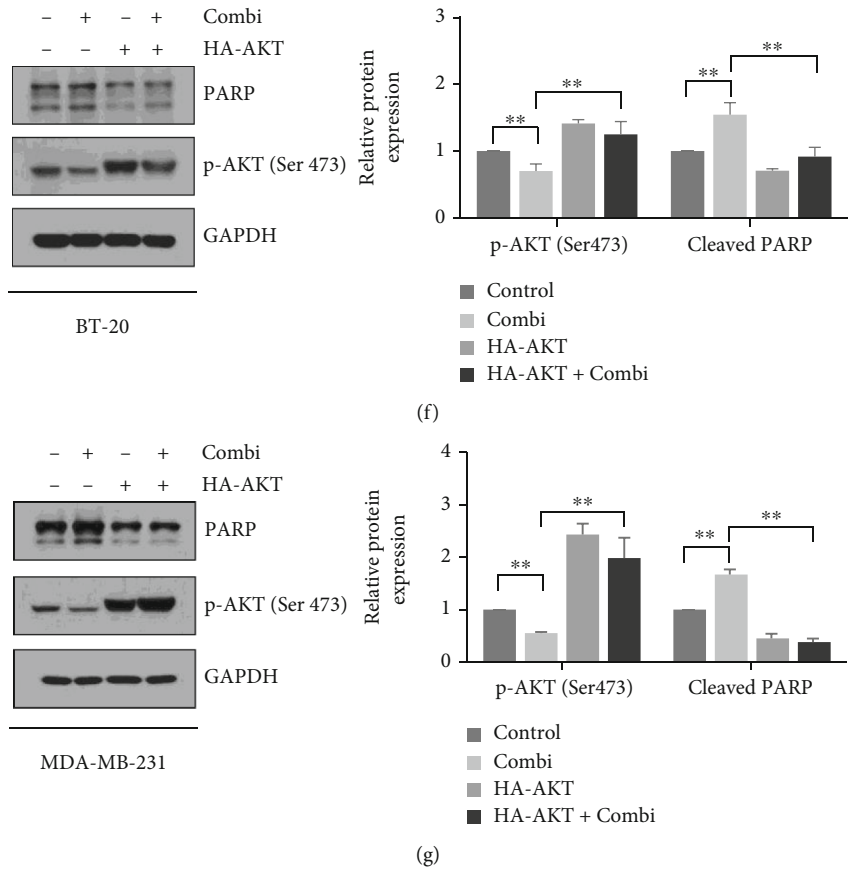

Figure 4: Combination treatment induces the apoptotic cell death through EGFR-AKT signaling suppression. Cells were pretreated with EGF (100 ng/mL) in serum-free media for 15 min and then treated with SH003 (100 μg/mL) and DTX (10 nM or 100 nM) for 24 h. (a) Cell viability was measured by MTT assay. (b, c) Cells were harvested with 2× sample buffer and indicated proteins were analyzed by western blotting. The bar graph indicates the relative protein expression and normalized to GAPDH. (d) After drug treatment for 24 h, cells were harvested and stained with Annexin V/7-AAD. Apoptosis was analyzed by flow cytometry. (e) Cells were transfected with pCDNA3-Myr-HA-AKT2 plasmid for 24 h. And then cells were treated with SH003 and DTX for 24 h. Cell viability was analyzed using MTT assay. (f, g) Apoptosis marker and the expression of AKT phosphorylation were detected by western blotting. The results represent mean ± SD on three independent experiments. $*P < 0.05$, $**P < 0.01$.

in vivo, we observed antitumor effect in BT-20 breast cancer xenograft mouse model. Combinatorial treatment of SH003 (557.57 mg/kg) and DTX (15.28 mg/kg) most effectively suppressed tumor growth (Figure 5(a)). Consistently, the combinatorial treatment reduced tumor weight and size (Figure 5(b)). However, those drugs did not affect body weight (Figure 5(c)).

Our immunohistochemistry confirmed that the combinatorial treatment effectively blocked tumor growth, as it reduced Ki67-positive cell numbers but increased cleaved caspase-3-positive cell numbers (Figure 6, second and third rows). Accordingly, the combinatorial treatment reduced CD31-positive cell numbers (Figure 6, the fourth row). Moreover, the combinatorial treatment reduced the number of cells expressing EGFR phosphorylation at Tyr 1068 (Figure 6, the fifth row).

4. Discussion

Although DTX has been used in treatment of many cancer patients, high dose of DTX induces severe side effects including anemia, peripheral neuropathy, and nausea. Moreover, low sensitivity and acquired drug resistance of DTX are still problems [38, 39]. For enhancing the effectiveness of DTX, studies about the combination therapy have been investigated in clinical models, especially in TNBC patients [40, 41]. In this study, we evaluated the synergistic effect of SH003 and DTX in TNBC cell lines and found that SH003 enhances the anticancer effect of DTX by inducing apoptosis and inhibiting the EGFR-AKT signaling pathway of TNBC *in vitro* and *in vivo* with no adverse effect (Figure 7).

In combination therapeutics with synthetic drugs, role of natural products is highly significant due either to a promotion in bioavailability and to a reduction of drug dose *via* synergistic effect [42]. Natural agents such as curcumin, 20S-protopanaxadiol, and flavonoids showed the combination effect with anticancer drugs by inhibiting cell proliferation or drug resistance mechanisms in breast cancer cells [43–45]. We have reported that SH003 has the anticancer effect and also synergistic effect with chemotherapy in TNBC [20, 24, 26]. Thus, the present study is aimed at

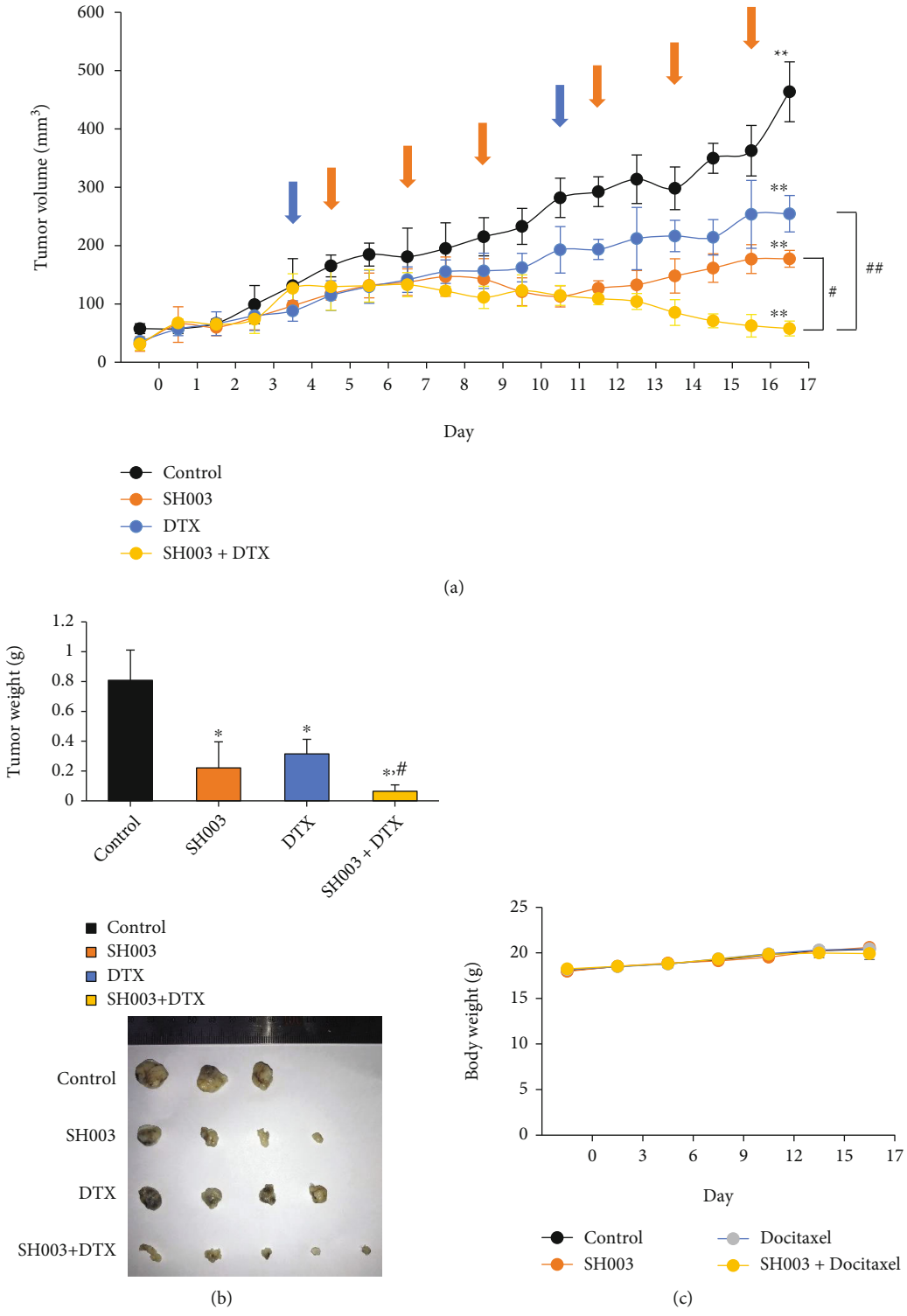

FIGURE 5: SH003 in combination with DTX enhances the tumor suppression in BT-20 xenograft mouse model. BT-20 cells (1×10^7 cells/100 μL PBS) were inoculated subcutaneously in the right flank of BALB/c nude mice. Mice were divided into four groups; control ($n = 3$), SH003 ($n = 4$), DTX ($n = 4$), and SH003 + DTX ($n = 5$). After tumor size was reached at about 100 mm^3, the mice were treated with indicated drugs. (a) DTX was administered at dose of 15.28 mg/kg once a week by intravenous injection (blue arrow). SH003 was treated orally at a dose of 557.57 mg/kg 3 times a week (orange arrow). Tumor volumes were measured using a caliper daily. $*P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. SH003 or DTX by one-way ANOVA with Bonferroni's post hoc test. (b) The graph shows tumor weight. $*P < 0.05$, $**P < 0.01$ vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. DTX by one-way ANOVA followed by Dunnett's test. (c) Body weight was measured twice a week. Data were expressed as the mean ± SD.

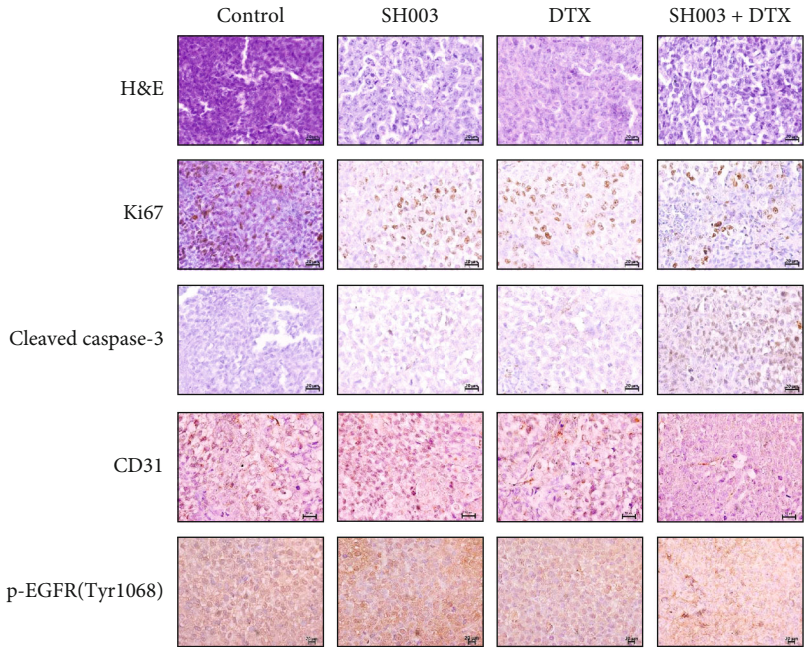

Figure 6: Histological analysis of BT-20 xenograft mouse model. Tumor tissues were stained with hematoxylin & eosin (H&E) and immunostained with Ki67, cleaved caspase-3, CD31, and p-EGFR (Tyr 1068). Scale bar indicates 20 μm.

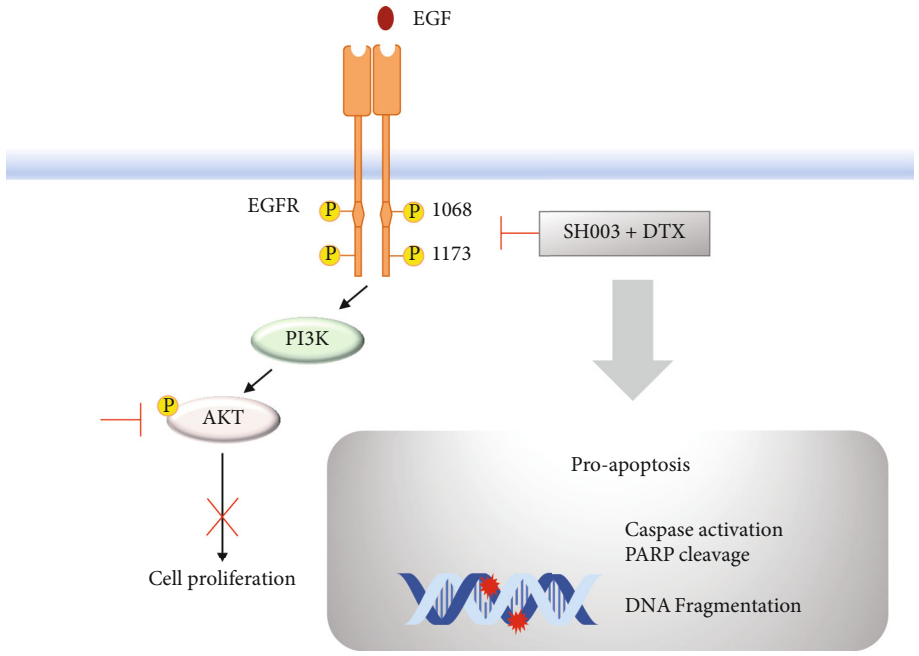

Figure 7: Schematic diagram of regulation mechanism by combined with SH003 and DTX on TNBC.

determining the highest effective dose of SH003 and DTX which has the synergistic effect against TNBC.

Combination therapy is the result of drug interactions, and it is important to find effective drug pairs. In particular, response rates to anticancer drugs in cancer cells could be different because of cancer heterogeneous characteristics, so an appropriate target dose of each drug in combination regimen should be determined. In addition, in order to apply to patients, it is necessary to determine the optimal combination dose through clinical trials [46]. Our results indicated the drug synergism at lower concentrations of SH003 and DTX in vitro, but this synergistic effect did not increase at higher concentrations of SH003. In particular, high dose of SH003 showed antagonistic action in BT-20 cells. But we observed that the combination therapy had antitumor efficacy at the dose used in clinical trial. By using

the concentrations of DTX (75 mg/m2) and SH003 (4800 mg/65 kg) determined by MTD and NOAEL studies, we validated the tumor suppression effect of combination treatment, minimizing the side effects in BT-20 cells. These results suggest that clinical studies should be conducted to find an appropriate dose combination regimen that works synergistically while reducing possible side effects. Thus, we demonstrated that SH003 can be a potential agent for combinatorial therapy with DTX, expecting to minimize DTX toxicity in clinical use.

Because high level of EGFR is discovered in about 40% of TNBC, EGFR is a promising target for treatment of TNBC. EGFR-targeted therapy has been developed and used to study for TNBC treatment. Currently, clinical trials of DTX combined with anti-EGFR antibody are ongoing in TNBC patients (NCT01939054) [47]. Although many clinical trials have attempted EGFR-targeting therapies in patient with TNBC subtype, most patients indicated no significant differences in overall response rate [6]. In case of NSCLC treatments, wild-type EGFR NSCLC patients also have low response to EGFR-TKI (tyrosine kinase inhibitors) treatment due to EGFR-TKIs are mostly effective in NSCLC harboring EGFR activating mutations [48]. These results suggest that the development of novel therapeutics targeting wild-type EGFR can overcome failure of EGFR-TKI therapy in TNBC [49]. Our study demonstrated that combinatorial treatment of SH003 and DTX significantly suppresses phosphorylation of EGFR *in vitro* and *in vivo* model. Thus, we suggest that this combinatorial treatment may be an effective therapy for wild-type EGFR amplified TNBC. Further studies are needed to confirm if SH003 and DTX treatment improves EGFR-TKI sensitivity in wild-type EGFR TNBC.

Many studies have shown that the dysregulation of EGFR signaling pathways including AKT, ERK and STAT3 is associated with tumor proliferation and survival [50–52]. Oncogenic function of these pathways contributes to chemoresistance and metastasis in TNBC and thus has been regarded as potential therapeutic targets [37, 53, 54]. We demonstrated that the combinatorial treatment induces apoptotic cell death by suppressing the activation of AKT in both BT-20 and MDA-MB-231 cells. SH003 combined with DTX significantly inhibited ERK and STAT3 phosphorylation in BT-20 cells while activity of ERK and STAT3 is not changed in MDA-MB-231 cells. In the recent study, it was reported that ERK is constitutively activated in MDA-MB-231 cells when compared with BT-20 cells [55]. Also, the expression of STAT3 protein was similar in BT-20 and MDA-MB-231 cells, but a level of STAT3 phosphorylation showed relatively low activity in MDA-MB-231 cells. These diversities of STAT3 activity can be caused by protein expression of ErbB family members as STAT3 upstream pathway [56]. In the therapy of cancer patients through EGFR inhibition, it has been reported that the regulation of the EGFR downstream proteins is important to determine the clinical response [57–59]. Taken together, the different response to combinatorial treatment in both cell lines can be explained by cell line-specific genetic characters. These results can support our finding that the combinatorial treatment more effectively inhibits the proliferation of BT-20 cells at low dose compared with MDA-MB-231 cells. In addition, it can be considered as a biomarker to determine the efficacy and reactivity of drugs depending on which EGFR downstream signaling is regulated.

5. Conclusions

The present study suggests that DTX in combination with SH003 would be a good strategy to treat TNBC patients. Furthermore, this combination reduced the EGFR activity and its downstream AKT signaling pathway as a major treatment target for EGFR-amplified TNBC. According to these observations, we also suggest that combination treatment of SH003 and DTX is possible to be considered as an effective drug combination strategy for the treatment of patients with TNBC.

Abbreviations

TNBC: Triple-negative breast cancer
DTX: Docetaxel
CI: Combination index
Am: *Astragalus membranaceus*
Ag: *Angelica gigas*
Tk: *Trichosanthes Kirilowii Maximowicz*
EGFR: Epidermal growth factor receptor
TKI: Tyrosine kinase inhibitor.

Data Availability

No data were used to support this study.

Conflicts of Interest

The authors declare no conflict of interest in this research.

Authors' Contributions

Y.J.C. and K.L. designed and carried out the experiments and drafted and revised the manuscript. S.G.C. participated in its design and revised the manuscript. J.H.Y., Y.G.K., M.J., H.H.H., S.Y.L., and S.E.J. performed experiments and data analysis. S.G.K. did the supervision and conceptualization. Yu-Jeong Choi and Kangwook Lee contributed equally to this work.

Acknowledgments

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Research Article

Antiinflammatory and Anticancer Properties of *Grewia asiatica* Crude Extracts and Fractions: A Bioassay-Guided Approach

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The study was an extension of our earlier work on antiinflammatory and anticancer properties of *G. asiatica* fruit. We aimed to develop a bioassay guided multistep purification technique for producing bioactive fractions of *G. asiatica* crude extracts. Dried fruit powder was sequentially fractionated with 100% dichloromethane, 100% methanol (MeOH), and 50% MeOH. Active extracts were subjected to liquid-liquid partitioning followed by subfractionation using RP-HPLC. Antioxidant, antiinflammatory, and anticancer activities of the fruit extracts, and their potent fractions were evaluated *in vitro*, while identification of compounds from the bioactive fractions was performed by ESI-MS/MS analysis. The amount of the identified compounds present was confirmed using external standards adopting a simple, accurate, and rapid analytical HPLC method. The results showed that 100% and 50% MeOH extracts possessed bioactivity; one of which (the 50% MeOH extract) displayed potent activity in all *in vitro* bioassays. MeOH extract (50%) derived fraction C and hydroalcoholic fraction 5 (GAHAF5) were observed to possess higher antioxidant, antiinflammatory, and *in vitro* anticancer activity. IC_{50} of GAHAF5 against MCF-7, HEp-2, and NCI-H522 cancer cells was recorded as 26.2, 51.4, and 63 μg/mL, respectively. ESI-MS/MS and HPLC analysis identified catechin, chlorogenic acid, caffeic acid, and morin as potential bioactive compounds in the GAHAF5 fraction with concentrations of 1230, 491, 957, and 130 μg/g, respectively. The findings indicated that *G. asiatica* bioactive fractions possessed antiinflammatory activity *in vitro* and were cytotoxic against breast cancer, lung cancer, and laryngeal cancer cell lines.

1. Introduction

Worldwide, an increasing rate of mortality linked to cardiovascular complications, diabetes, various forms of cancer, and many other physiological ailments is creating a burden on the healthcare systems. A plethora of literature correlate modifiable lifestyle factors or health behaviors, for example, poor dietary choices, tobacco use, excessive intake of alcohol, and physical inactivity, with an increasing incidence of the aforementioned chronic diseases [1, 2]. Such a situation calls for a paradigm shift in approaches to improving the health of individuals and hence populations [3]. Fruits and vegetables contain nutrient and nonnutrient substances bearing medicinal properties and are traditionally widely accepted for their relevance to the prevention of various health ailments. However, uncertainties in links between consumption of fruits and vegetables and the reduction of risk of chronic disease require the systematic establishment of a clear connection between the nutrient or nonnutrient components and their ability to reduce the risk of disease [4].

Cancer is among the most common of life-threatening diseases. The International Agency for Research on Cancer has reported worldwide an estimation of 19.3 million new

cancer cases and almost 10.0 million cancer-related deaths in the year 2020 [5]. According to the Global Cancer Statistics, Asia has the highest share of cancer-related deaths (57.3%). Approximately one-half of all new cancer cases and more than one-half of the overall cancer deaths were reported from Asia. With an estimation of 2.26 million new cases, breast cancer was reported as the leading type of cancers diagnosed, while the highest incident of cancer-related mortalities, i.e., 1.79 million, was accounted for lung cancer [5, 6]. Chemotherapy, radiotherapy, and chemical drugs are commonly utilized cancer therapies; however, the cost of treatment is high, and there are often adverse side effects [7, 8]. For these reasons, plant-based new medicines are being increasingly investigated as possible alternatives to synthetic drugs subject to their efficacy and consumers' safety [9].

More than 60% of anticancer drugs including vinblastine, vincristine, camptothecin, taxol, podophyllotoxin, and combretastatin are reported to be retrieved from natural sources [10]. The importance of plants and fungi is illustrated by the discovery of penicillin, morphine, and aspirin, the semisynthetic acetylated derivatives of natural salicylic acid. Penicillin is an antibiotic, morphine is a pain reliever, and aspirin is a cyclooxygenase inhibitor used as an antipain, antifever, and antiinflammatory drug, as well as in the prevention of strokes and heart attack owing to its antiplatelet potential [11, 12]. However, many plants used in conventional medicinal or healthcare systems still require scientific exploration and validation.

G. asiatica L. (*Tiliaceae*), a berry indigenously known as *Phalsa*, is endemic to South Asian countries, mainly Pakistan and India [1]. Ethnic or traditional use of *G. asiatica* fruit is predominantly used as a refreshing, thirst quenching drink in summer. The fruit drink, also known in some countries as "phalsay ka sharbat," is thought to be tonic, while the fruit and bark infusions of *G. asiatica* are considered demulcent, febrifuge, and diarrheal remedies [12]. The traditional medicinal claims of *G. asiatica* and its secondary metabolites like flavonoids, phenolic acids, and anthocyanins are supported by the documented pharmacological activities, including antiinflammatory, antidiabetic, anticancer, skin health promotive, and reduction of risk of coronary heart disease [13–16].

In an extension of our earlier work "anticancer and antiinflammatory perspectives of Pakistan's indigenous berry *Grewia asiatica* Linn (*Phalsa*)" [16], there was a need to identify bioactive fractions of *G. asiatica* as a prelude to their potential exploitation in the food and pharmaceutical industry. This study was therefore intended to develop a bioassay-guided sequential extraction method for recovering bioactive rich fractions of *G. asiatica* fruit and to isolate fractions that may possess higher free radical scavenging, antiinflammatory, and *in vitro* cytotoxic activities against human cancer cells.

2. Materials and Methods

2.1. Plant Material. Grewia asiatica fruits known in Pakistan as *Phalsa* were supplied by a local grower (Multan, Punjab, Pakistan), and taxonomic identification was performed by a botanical expert from the Department of Botany, Bahauddin Zakariya University, Multan. The berries were washed to remove dirt, and then the seed-free pulp was extracted with a fine fruit pulper. The fruit pulp was spread onto stainless steel trays with thickness lesser than 4 mm and dehydrated in a commercial dehydrator (Pamico Tech. Pak) at 40°C. The dehydrated fruit pulp was ground to powder in a kitchen scale grinder and stored in an airtight jar at 4°C for further processing.

2.2. Solvents and Reagents. HPLC grade solvents (water, methanol), antiinflammatory and anticancer standard drugs (diclofenac sodium, methotrexate), and analytical and preparative HPLC columns (Zorbax-SB-C-18, Agilent) were purchased from the local supplier of Sigma-Aldrich, USA. Analytical grade solvents, antioxidant assays' reagents, and reference standards were procured from Merck, Pakistan.

2.3. Ethical Approval. All studies and testing protocols were in line with the ethical codes mentioned in the Declaration of Helsinki, duly approved by the Bioethical Committee, Bahauddin Zakariya University, Multan, Reg. no. 05-18.

2.4. Preparation of Extracts. The dried fruit powder was delipidated with hexane and filtered through Whatman No. 1 filter paper. Filtration residues were sequentially fractionated with 100% dichloromethane (DCM), 100% methanol (MeOH), and then 50% MeOH (50 : 50 v/v H_2O : MeOH) using an orbital shaker. All filtrates were subjected to rotary evaporation (Heidolph, Germany) and stored in an upright ultralow freezer (Sanyo, Japan) at −40°C for bioactive fraction screening assays. The most potent extracts, as evaluated by performance of bioassays, were subjected to liquid-liquid partitioning by solubilization with water (Fraction C) and then partitioned successively, first with chloroform (Fraction A) and then with ethyl acetate (Fraction B), as illustrated in Figure 1.

2.5. Determination of Antioxidant Activity. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of the test samples was conducted in accordance with the method proposed by Alara et al. [17]. The results were calculated as IC_{50} (μg/mL) and compared with the standards, i.e., ascorbic acid and quercetin.

Similarly, hydrogen peroxide (H_2O_2) scavenging ability assay was performed with the method followed by Ruch et al. [18] with minor changes. Ascorbic acid and quercetin were used as reference standards, and the results are computed using the following equation:

$$\%\text{inhibition} = \frac{(Ao - As)}{Ao} \times 100 \quad (1)$$

where A_o indicates the absorbance measured for control and A_s refers to the sample or standard absorbance.

Ferric reducing antioxidant power (FRAP) assay was also performed in accordance with the method followed by

Zahin et al. [19]. Ferrous sulfate was used as a reference standard, and the results were computed as mM Fe/g.

2.6. In Vitro Antiinflammatory Activity Assays

2.6.1. Membrane Stabilization Assay (Heat-Induced Hemolysis). Blood was collected in heparinized tubes from healthy human subjects from the cubital vein and centrifuged at 1100 × g for 5 minutes. Blood cells were washed three times with normal saline. The volume of blood was measured and reconstituted as 10% v/v suspension with isotonic buffer solution (10 mM sodium phosphate buffer, pH 7.4) [20, 21].

The membrane stabilization assay was performed in accordance with the method described by Shinde et al. [22]. Briefly, a reaction mixture (2 mL) was prepared by adding 1 mL experimental extracts of different strengths, i.e., 50, 100, 200, and 300 μg/mL to 1 mL (10%) red blood cell (RBC) suspension in isotonic buffer solution (pH 7.4). The tubes containing the reaction mixture were incubated (25 min; 50°C) and cooled. The reaction mixture was centrifuged (1100 × g; 3 min), and the absorbance of the supernatant was checked at 560 nm using a spectrophotometer. Diclofenac sodium was used as a positive control while phosphate buffer as a negative control. The inhibition rate is calculated in accordance with the following equation:

$$\text{Inhibition}\,(\%) = \frac{(\text{Abs Control} - \text{Abs treated})}{\text{Abs Control}} \times 100. \quad (2)$$

2.6.2. Egg Albumin Denaturation Assay. Egg albumin denaturation assay was performed by the method described by Mizushima and Kobayashi [23]. Briefly, 5 mL of the reaction mixture was prepared by mixing egg albumin (0.2 mL), 2.8 mL phosphate buffer (pH 6.5), saline, and 2 mL of extracts of different concentrations, i.e., 50, 100, 200, and 300 μg/mL. The reaction mixture was incubated for a period of 20 min at 40°C followed by heating at 75°C for 5 min in a water bath. The contents of the tubes were cooled, and the absorbance was read spectrophotometrically at 660 nm. Diclofenac sodium and phosphate buffer solution were used as positive and negative control, respectively. Inhibition rates (%) are derived from the following equation:

$$\text{Inhibition}\,(\%) = \frac{(\text{Abs Control} - \text{Abs treated})}{\text{Abs Control}} \times 100. \quad (3)$$

2.6.3. Bovine Serum Albumin Denaturation Assay. Bovine serum albumin denaturation assay was performed in accordance with the method followed by Sakat et al. [21]. Reaction mixture (0.5 mL) was made by adding 0.45 mL of bovine serum albumin and 0.05 mL of experimental extracts of different strengths (50-300 μg/mL). Phosphate buffer (2.5 mL; pH 6.3) was mixed with the reaction mixture, and the contents of the test tubes were incubated for 25 min at 40°C. Cooling was performed, and absorbance was recorded with UV-Vis spectrophotometer (660 nm). Diclofenac sodium and phosphate buffer solution were used as positive and negative controls, respectively. The percent inhibition rates are calculated using the following equation:

$$\text{Inhibition}\,(\%) = \frac{(\text{Abs Control} - \text{Abs treated})}{\text{Abs Control}} \times 100. \quad (4)$$

2.7. In Vitro Anticancer Activity

2.7.1. Methyl Thiazolyl Tetrazolium (MTT) Assay. G. asiatica extracts and their fractions were evaluated for *in vitro* anticancer potential with the method utilized by Roy et al. [24]. Experimental samples of varying strengths (0.5–200 μg/mL) were prepared in 100 μL dimethylsulphoxide (1% v/v) in microtiter plates. After incubating the microtiter plates (37°C, 48 hours), to each well was added 50 μl of the MTT solution (5 mg/mL). A microplate reader (Tecan, Austria) was used to check the reduction in MTT after a second incubation (37°C, 4 hours) by recording the absorbance at 570 nm. The untreated cells were used as a control against which to measure the effect of experimental extracts on the cell viability. The percent inhibition exhibited on the cell cultures by the test samples is computed using the following equation:

$$\text{Survival}\,(\%) = \frac{(At - Ab)}{(Ac - Ab)} \times 100, \quad (5)$$

where At, Ab, and Ac indicate the sample, blank (complete media without cells), and control absorbance, respectively.

$$\text{Cell inhibition}\,(\%) = 100 - \text{cell survival}\,(\%). \quad (6)$$

2.8. Method Optimization for Fractionation Using RP-HPLC. Fraction C (50% MeOH extract) and Fraction B (100% MeOH extract) were further fractionated using RP-HPLC by dissolving solidified fractions in MeOH (100%) as suggested by Cock [25]. Method optimization for fractionation was performed through Agilent LC technology using an SB-C-18 analytical column (4.6 × 150 mm, 5 μm, Agilent, Germany). The sample was prepared in MeOH at the concentration of 10 mg/mL, and the contents were filtered using 0.45 μm syringe filter. The sample injection limit was set at 5 μL with a flow rate of 0.5 mL/min. Maximum peaks were recorded with acidified (0.1% TFA) water (A) and acidified (0.1% TFA) methanol (B) at 210 (Fraction C) and 280 nm (Fraction B).

2.9. RP-HPLC Fractionation (Reverse Phase Chromatography). RP-HPLC fractionation was performed using Zorbax SB-C18 semipreparative column (25 × 250 mm, 5 μm particle size, Agilent, Germany). The samples were prepared in 100% MeOH as 50 mg/mL; the injection limit was set as 1 mL with a flow rate of 10 mL/min. Eight subfractions were obtained from Fraction C (50% MeOH extract) named *G. asiatica* hydroalcoholic extracts, fractions 1–8, i.e., GAHAF1, GAHAF2, GAHAF3, GAHAF4, GAHAF5, GAHAF6, GAHAF7, and GAHAF8. Accordingly, 5 subfractions were derived from the Fraction B (100% MeOH extracts) termed as *G. asiatica* methanolic fractions 1–5, i.e., GAMF1, GAMF2, GAMF3, GAMF4, and GAMF5.

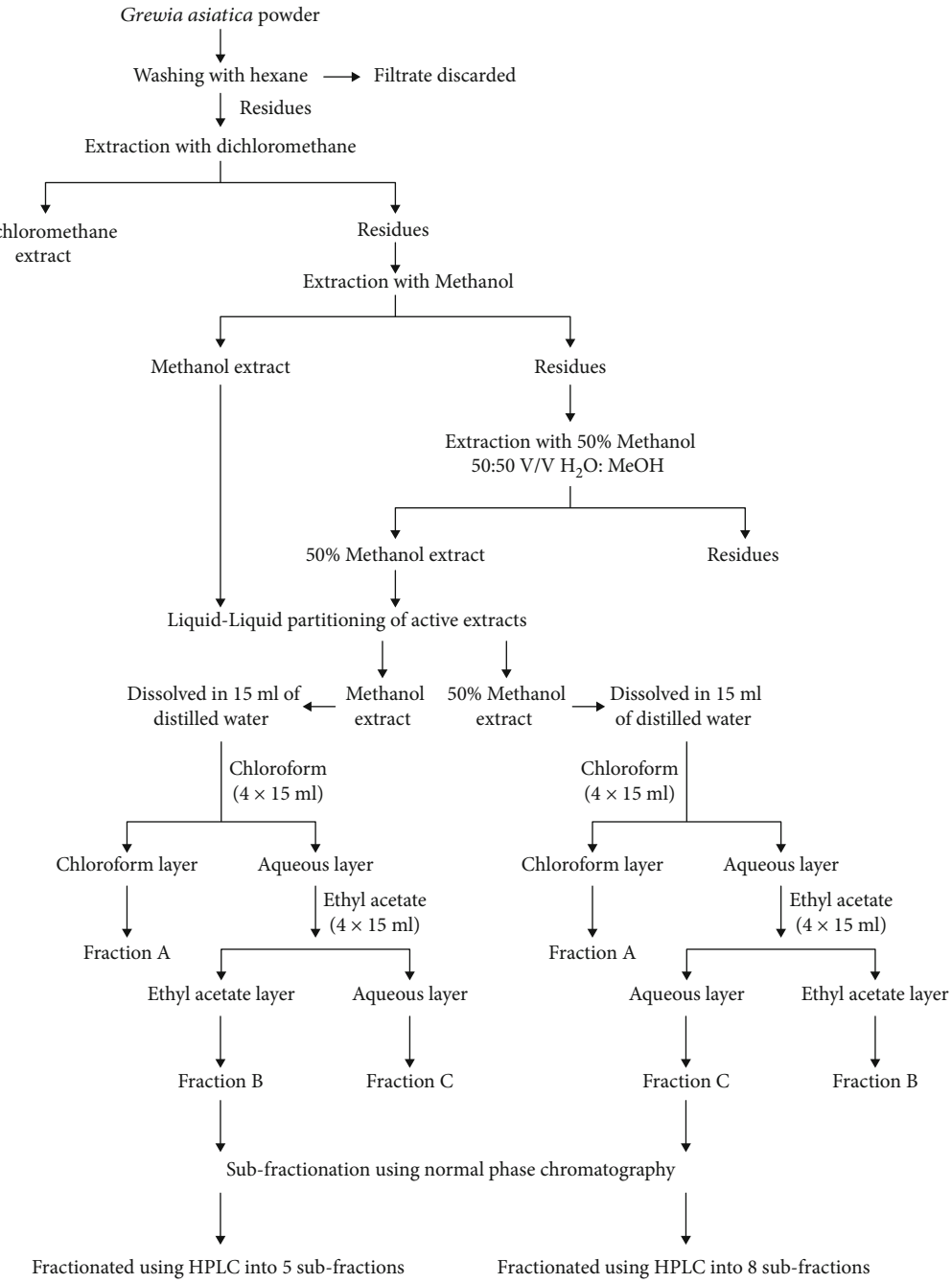

Figure 1: Schematic diagram of *G. asiatica* extract after alcoholic extraction, liquid – liquid partitioning, and subfractionation.

2.10. ESI-MS/MS Analysis. HPLC subfractions with potent activities led to LC-ESI-MS/MS (LTQ XL, Thermo Electron Corporation, USA) analysis for identification of bioactive components adapting guidelines as described earlier by Steinmann and Ganzera [26]. The online software (http://www.chemspider.com/) was used to obtain the structural details of the bioactive compounds identified in the present study.

2.11. Quantification Using External Standards. Bioactive compounds tentatively identified earlier were further confirmed by comparing the retention times with external standards and quantified from the percent peak area using HPLC. The chromatograms were obtained at different wavelengths (230, 254, 280, 300, 330 nm). The analytical method for qualitative and quantitative analysis was validated according to the guidelines issued by the proceedings of the International Conference on Harmonization (ICH) for specificity, linearity, accuracy, precision, LOD, and LOQ.

2.12. Statistical Analysis. The data were expressed as mean ± SD. One-way analysis of variance (ANOVA) was used to measure statistical differences between the controls and treatments. The data was subsequently analyzed by

Table 1: *In vitro* antioxidant and antiinflammatory potential of *G. asiatica*.

Fruit samples	*In vitro* antioxidant assays			*In vitro* antiinflammatory assays (% inhibition at 300 μg/mL)			Anticancer assay ($IC_{50}\mu$g/mL)		
	DPPH ($IC_{50}\mu$g/mL)	FRAP (mmol/g)	H_20_2 (%)	Heat-induced hemolysis	Egg albumin denaturation	Bovine serum albumin denaturation	MCF-7	HEp-2	NCI-H522
100% DCM	153 ± 2.3	14 ± 0.2	33 ± 1.44	12 ± 0.2^{ns}	17 ± 0.2^{ns}	21 ± 0.3^{ns}	175	257	232
100% MeOH	77 ± 1.1	27 ± 0.7	43 ± 0.4	32 ± 0.1*	36 ± 0.1*	44 ± 1.1*	86	126	107
Fraction B	62 ± 0.2	39 ± 0.9	51 ± 1.1	39 ± 0.1*	44 ± 0.9*	49 ± 1.1*	61	141	95
GAMF3	56 ± 0.3	34 ± 0.2	49 ± 0.1	46 ± 1.1*	51 ± 0.1*	56 ± 0.3**	52	162	67
50% MeOH	41 ± 1.0	43 ± 0.6	73 ± 0.6	50 ± 0.2*	56 ± 0.0**	65 ± 0.0**	35	80	73
Fraction C	37 ± 1.2	44 ± 0.2	70 ± 0.1	56 ± 0.1**	61 ± 0.2**	70 ± 0.2**	30	73	81
GAHAF5	29 ± 0.1	46 ± 1.1	77 ± 0.2	59 ± 0.2**	63 ± 1.2**	75 ± 1.1***	26	51	63
Ascorbic acid	21 ± 0.2	56 ± 1.1	78 ± 0.2			—			
Quercetin	18 ± 0.5	63 ± 0.1	82 ± 0.3			—			
Diclofenac sodium		—		98 ± 0.02****	96 ± 0.02****	98 ± 0.01****		—	
Methotrexate					—				

Values are means ± S.D. DCM extract = 100% dichloromethane extract; 100% MeOH extract = 100% methanolic extract; GAMF = *G. asiatica* methanolic fraction; 50% MeOH extract = methanol : water (50 : 50 v/v); GAHAF = *G. asiatica* hydroalcoholic fraction; DPPH = 2,2-diphenyl-1-picrylhydrazyl; FRAP = ferric reducing antioxidant power; TPTZ = 2,4,6-tripyridyl-s-triazine; H_2O_2 = hydrogen peroxide $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $***$ $*p < 0.0001$.

Dunnett's test. Prism (Graph Pad Software, San Diego, USA) was used to plot the graphs, and p values were indicated as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $***$ $*p < 0.0001$.

3. Results and Discussion

3.1. Antioxidant Activity. The results presented in Table 1 show that Fraction C and Fraction B possessed the lowest IC_{50} in the DPPH assay, the highest ferric reducing antioxidant power (FRAP), and the maximum radical scavenging in H_2O_2 assay compared to their parent extracts, i.e., 50% MeOH and 100% MeOH extract, respectively. Among RP-HPLC subfractions, GAHAF5 of Fraction C (50% MeOH extract) exhibited considerable radical scavenging potential in DPPH (IC_{50} of 29 μg/mL), FRAP (46 mmol/g), and H_2O_2 (77%) assays, comparable to the antioxidant activity of standard ascorbic acid or quercetin (Table 1). Likewise, a notable increase in the antioxidant activity was also observed for GAMF3 in comparison to Fraction B (100% MeOH extract). Previously, a methanol extract of *G. asiatica* leaves was reported to hold notable DPPH radical scavenging activity (IC_{50} of 27.3 pg/mL) and nitric oxide radical inhibition activity (IC_{50} of 56.85 μg/mL) when compared with ascorbic acid or quercetin as standards [27]. The strong antioxidant potential of *G. asiatica* was also cited by Mesaik et al. [28], who reported that the 20 ppm flavanol fraction of the fruit produced 85% inhibition of DPPH stable-free radicals. Data on qualitative screening of the *G. asiatica* extracts (Supplementary Table 1) further suggest the fruit as plausible carrier of secondary metabolites such as flavonoids, phenols, and tannins which may serve as active-free radical inhibitors alike other fruits of ethnomedicinal significance including *S. cumini* and *C. carandas*.

3.2. In Vitro Antiinflammatory Activity

3.2.1. Heat-Induced Hemolysis (Membrane Stabilization). The stabilization effect of a drug on human red blood cell membranes (HRBC) against heat and hypotonicity induced lysis is documented as a proxy for antiinflammatory action of a drug [29, 30]. The human red blood cell membrane and lysosomal membrane are similar in composition; therefore, drugs protecting HRBC membrane may also protect against destruction of the lysosomal membrane [31].

Consistent with the above, antiinflammatory activity of *G. asiatica* successive extracts, their partitioned fractions, and RP-HPLC subfractions were studied at varying concentrations (50, 100, 200, and 300 μg/mL). The findings revealed a dose-dependent inhibition of heat-induced hemolysis and improved efficacy of the extracts from crude RP–HPLC subfractions. Among the tested fractions, GAHAF5 anticipated 59% inhibition of heat-induced hemolysis at a dose of 300 μg/mL, whilst the inhibition by 50% MeOH extract and fraction C was 50 and 56%, respectively. Data shown in Table 1 suggest a strong relationship between antioxidant and antiinflammatory activity. A similar study conducted by Moussaid et al. [32] reported a positive correlation between the concentrations of experimental extracts and membrane stabilization. Furthermore, extracts having antioxidant activity considerably inhibited the inflammatory responses. Likewise, 100% MeOH extract, Fraction B, and GAMF3 dispensed at 300 μg/mL exerted 32 and 46% inhibition in heat-induced hemolysis when compared with the normal control, respectively. Earlier, Khanal et al. [33] in their study

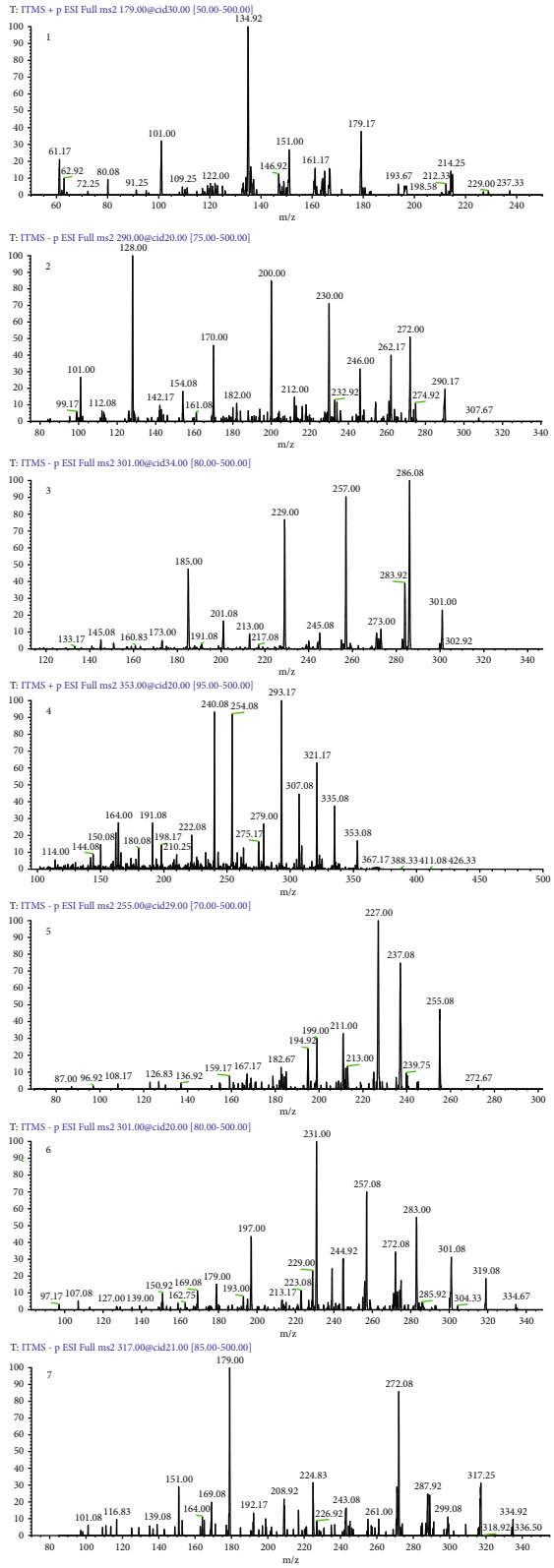

Figure 2: Mass spectras of identified compounds. (1) Caffeic acid; (2) catechin; (3) morin; (4) chlorogenic acid; (5) liquiritigenin; (6) quercetin; (7) myricetin.

on antiinflammatory activity of *G. asiatica* fruit extracts—as determined with membrane stabilization assays—reported 81% inhibition at 600 μg/mL, a finding remarkably like ours.

3.2.2. Inhibition of Protein Denaturation (Serum and Egg Albumin). Denaturation of proteins is a well-documented cause of inflammation, while substantial data is available to confirm the link between denaturation of tissue proteins and the onset of inflammatory complications [34, 35]. Plant extracts, owing to their capacity to react with erythrocyte membrane proteins, may deform the cells [21] and provoke alteration of cell surface charges [36].

Our multistep purification technique produced fractions that positively affected egg albumin denaturation inhibition: indeed, a 56% inhibition of albumin denaturation was observed for crude extracts (50% MeOH extract), and a 63% inhibition was found in the case of the purified successive fraction (GAHAF5). The results further suggested a dose-dependent albumin denaturation inhibitory response that has also been documented for coffee extracts compared with the diclofenac sodium standard [37]. Another study delineated a dose-dependent effect of methanolic extract of *Enicostemma axillare* with a maximum inhibition of 71% observed at 500 μg/mL [38]. Accordingly, moderate inhibition of protein denaturation at 300 μg/mL was observed with pure MeOH extract (36%), Fraction B (44%), and GAMF3 (51%) (Table 1).

A similar inhibitory trend was recorded against serum albumin denaturation assay suggesting that 50% MeOH extract (300 μg/mL) exhibit the highest inhibition of albumin denaturation, i.e., 65%, when compared to other extracts. Among liquid-liquid partitioned fractions, only Fraction C and, from RP-HPLC subtractions, only GAHAF5 outlined inhibitory activity as 70% and 75%, respectively, in albumin denaturation inhibition assay which is slightly more than the parent 50% MeOH extract. A standard drug, diclofenac sodium, offered a potent inhibition of 98%, 96%, and 98% at a dose of 300 μg/mL against heat-induced hemolysis, egg albumin denaturation, and bovine serum albumin denaturation assays, respectively (Table 1).

3.3. Anticancer Activity. The crude MeOH extracts (50 and 100%) demonstrated potent to moderate *in vitro* anticancer activity which was further characterized in the successive extracts obtained through bioassay guided fractionation. Bioassay-guided fractionation is an established and effective method for purifying anticancer, antifungal, and antifertility compounds [39–41]. Our results suggest that the 50% MeOH extract derived Fraction C as the fraction exhibiting the highest anticancer activity, with an IC_{50} of 30 μg/mL, 81 μg/mL, 73 μg/mL, 114 μg/mL, and 279 μg/mL against human cell lines derived from breast cancer, lung cancer, laryngeal cancer, epidermal kidney cancer, and cervical cancer, respectively. Since IC_{50} of extracts and fractions against epidermal kidney cancer and cervical cancer were above 100 μg/mL, the values are therefore not tabulated. In the case of breast and laryngeal cancer cell lines, Fraction C demonstrated the lowest IC_{50} when compared with the parent extracts. Similarly, Fraction B originating from 100% MeOH extract exerted considerable cytotoxic activity against breast

Table 2: ESI-MS/MS identification of bioactive compounds from *G. asiatica* different fruit fractions.

Fractions	Average mass	ESI-MS/MSn (ions)	Identification	Chemical formula	References
GAHAF5	179	179, 161	Caffeic acid	$C_9H_8O_4$	[62]
	290	290, 272.08, 246	Catechin	$C_{15}H_{14}O_6$	[62]
	302	302, 286.07	Morin	$C_{15}H_{10}O_7$	[61]
	354	353.25, 191	Chlorogenic acid	$C_{16}H_{18}O_9$	[62]
GAMF3	256	255, 237	Liquiritigenin	$C_{15}H_{12}O_4$	[63]
	301	301, 272, 179	Quercetin	$C_{15}H_{10}O_7$	[64]
	317	317, 179	Myricetin	$C_{15}H_{10}O_8$	[64]

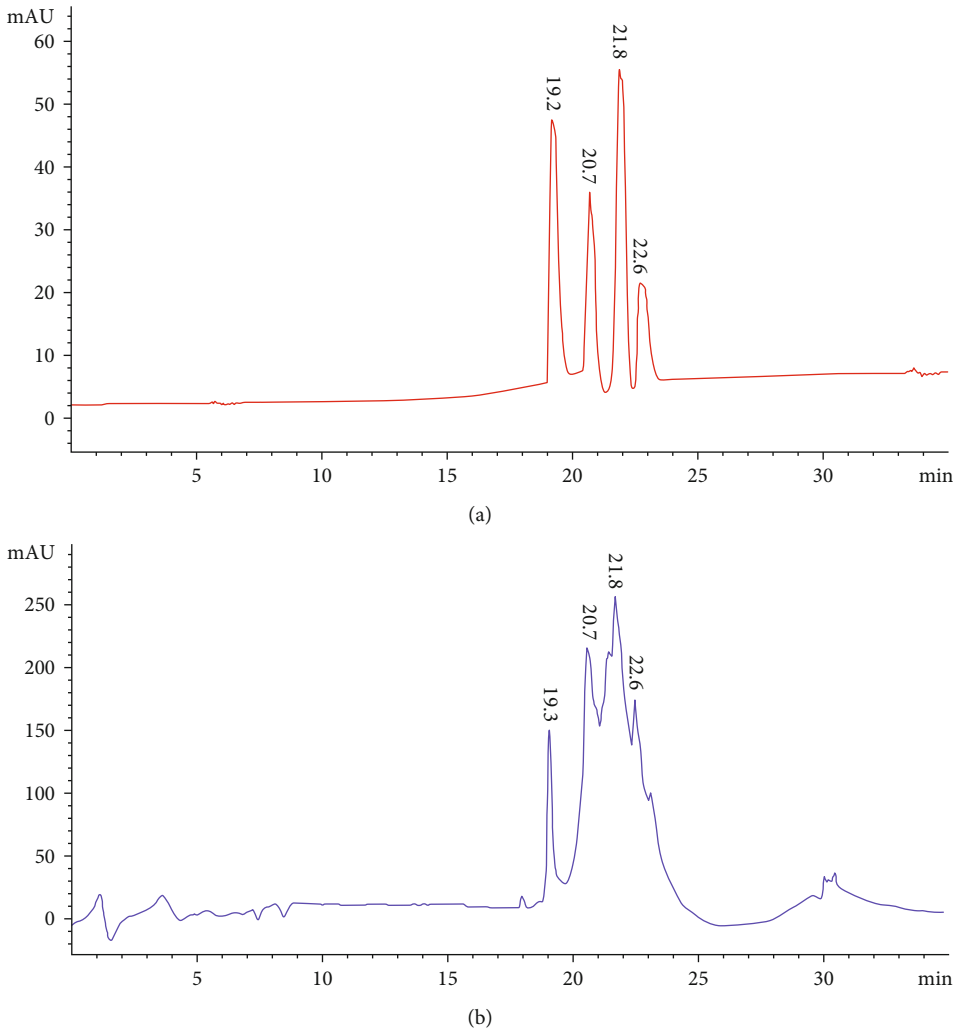

Figure 3: HPLC chromatograms of external standards (A) and GAHAF5 (B) at 300 nm. (1)Catechin; (2) chlorogenic acid; (3) caffeic acid; (4) morin.

and lung cancer cell lines. IC_{50} of fraction B against breast cancer and lung cancer were 61 μg/mL and 95 μg/mL, respectively, which are also higher than recorded from the parent extracts (Table 1).

The IC_{50} for 50% MeOH extract against MCF-7 breast cancer cells was up to 35 μg/mL, while lower values were recorded for fraction C and subfraction GAHAF5, i.e., 30 μg/mL and 26 μg/mL, respectively. Parallel trends were observed with lung and laryngeal cancer cell lines, where GAHAF5 exhibited the lowest IC_{50}, i.e., 63 μg/mL and 51 μg/mL with lung cancer and laryngeal cancer, respectively. Interestingly, GAMF3 from Fraction B was cytotoxic against the breast cancer cell line (IC_{50} of 52 μg/mL). Previously, M1 fractions retrieved from the parent hexane extract of *Mangifera zeylanica* bark obtained through a series of purification techniques were reported more effective than

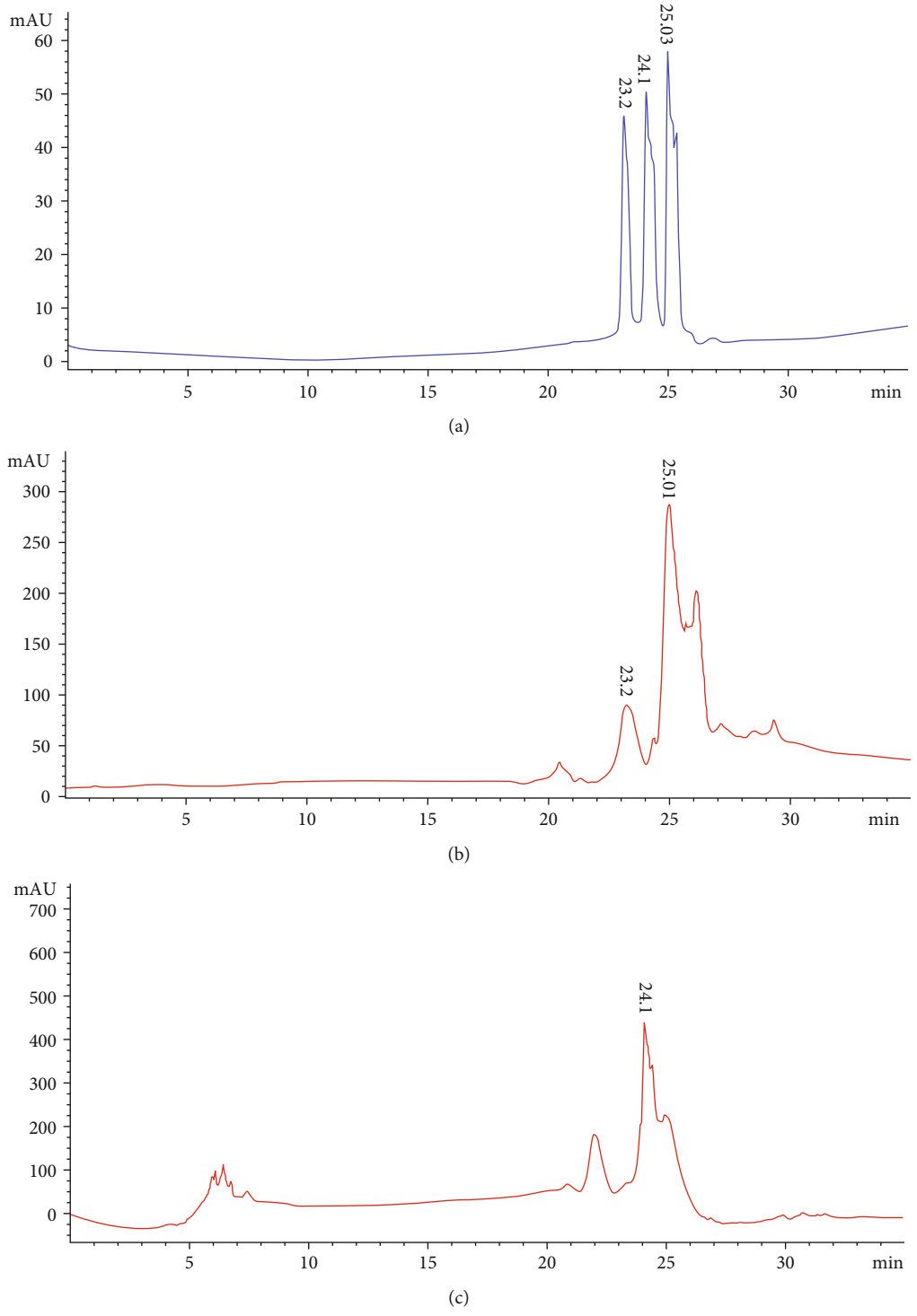

Figure 4: HPLC chromatograms of external standards (a) and GAMF3 at 230 nm (b) and at 230 nm (c): (1) liquiritigenin; (2) quercetin; (3) myricetin.

the parent hexane extract against three cancer cell lines. The referenced study demonstrated a less toxic effect of bioassay guided fraction when compared with the parent extracts on normal mammary epithelial cells [41]. Glaucarubinone, a triterpenoid obtained from the hexane extracts of Brazilian cerrado, was reported biologically active against cancer cell lines of common cancers [42].

3.4. ESI-MS/MS Analysis. The bioactive fraction of *G. asiatica* fruit extracts, i.e., GAHAF5, was identified to contain caffeic acid, morin, catechin, and chlorogenic acid (Figure 2. Table 2). Mass spectras of compounds that we were unable to identify are also available in Supplementary Figures 1 and 2. Previously, all referred compounds have been cited as antiinflammatory agents [43–46]. Morin

Table 3: Quantification parameters of three phenolic acids, and four flavonoids from *G. asiatica* different fruit fractions.

Fractions	Compounds name	Wavelength	LOD	LOQ	Linear range (μg/mL)	r^2	R_t min	Concentration (μg/g)
GAHAF5 (50% MeOH extract)	Catechin	300 nm	3.10	9.60	46.8-1500	0.9994	19.3	1230
	Chlorogenic acid		0.2	0.5	7.8-500	0.9995	20.7	491
	Caffeic acid		2.7	8.4	7.8-500	0.9991	21.8	957
GAMF3 (100% MeOH extract)	Morin		1.3	3.24	46.8-1500	0.9993	22.6	130
	Myricetin	330 nm	0.9	2.16	12.5-200	0.9985	24.1	217
	Quercetin	230 nm	1.1	2.75	1.6-25	0.9991	25.0	591
	Liquiritigenin		2	4.90	3.9-250	0.9987	23.2	24

LOD = limit of detection; LOQ = limit of quantification; r^2 = regression coefficient. Rt min = retention time in minutes; GAMF3 = *Grewia asiatica* methanolic fraction; GAHAF5 = *Grewia asiatica* hydroalcoholic fraction; 100% MeOH = 100% methanolic extract; 50% methanolic extract (50/50 H_2O: MeOH).

Table 4: Accuracy validation of analytical method three phenolic acids and four flavonoids from *G. asiatica* different fruit fractions.

Marker substance	Standard additions μg/mL	% recovery Day 1	Day 2	Day 3	Mean	% CV
Catechin	200	97.1	97.9	99.4	98.12 ± 1.16	1.18
	400	101.2	98.7	100.7	100.2 ± 1.32	1.31
	500	101.3	102.9	100.2	101.4 ± 1.35	1.33
Chlorogenic acid	200	97.2	97.9	99.1	98.06 ± 1.01	1.02
	400	99.3	99.8	102.4	100.5 ± 1.16	1.15
	500	103.4	101.4	102.9	102.5 ± 1.04	1.01
Caffeic acid	200	101.3	99.1	104.3	101.5 ± 2.61	2.57
	400	103.5	104.3	100.5	102.7 ± 2.00	1.94
	500	100.5	103.3	105.6	103.1 ± 2.55	2.47
Morin	200	97.4	98.0	100.5	98.63 ± 1.64	1.66
	400	99.9	97.3	101.2	99.46 ± 1.98	1.99
	500	100.9	103.4	105.3	103.2 ± 2.20	2.13
Quercetin	200	99.0	103.3	97.8	100.03 ± 2.89	2.88
	400	98.9	101.4	96.2	98.83 ± 2.60	2.63
	500	104.5	105.4	102.4	104.1 ± 1.53	1.46
Myricetin	200	96.0	100.3	101.8	99.36 ± 3.01	3.02
	400	99.9	102.4	97.2	99.83 ± 2.60	2.60
	500	99.5	101.4	104.4	101.7 ± 2.47	2.42
Liquiritigenin	200	98.0	100.3	96.8	98.36 ± 1.77	1.79
	400	97.9	100.4	105.2	101.1 ± 3.70	3.65
	500	100.5	103.4	102.4	102.1 ± 1.47	1.43

Values shared are mean ± SD of triplicates. Percent coefficient of variation (% CV); (SD/Mean) × 100.

appeared to induce cytotoxicity among metastatic breast and lung cancer cells [47, 48]. Caffeic acid was reported to induce apoptosis in breast cancer [49] and lung cancer cells [50]. Chlorogenic acid regulated apoptosis in A549 human lung and breast cancer cells [51]. In addition, catechin was reported to exhibit a significant inhibition in the proliferation of breast [52] and lung cancer [53].

Likewise, liquiritigenin, quercetin, and myricetin were also identified in GAMF3 fraction of our study (Figure 2), while the literature confirms the significant antiinflammatory potential of liquiritigenin [54], myricetin [55], and quercetin [56]. Moreover, liquiritigenin, myricetin, and quercetin were also presented as potent breast cancer inhibitors in some previous findings [57–59].

3.5. Quantification of Bioactive Compounds Using External Standards

3.5.1. Specificity Validation. The analytical method was evaluated for its specificity by comparing the retention times and

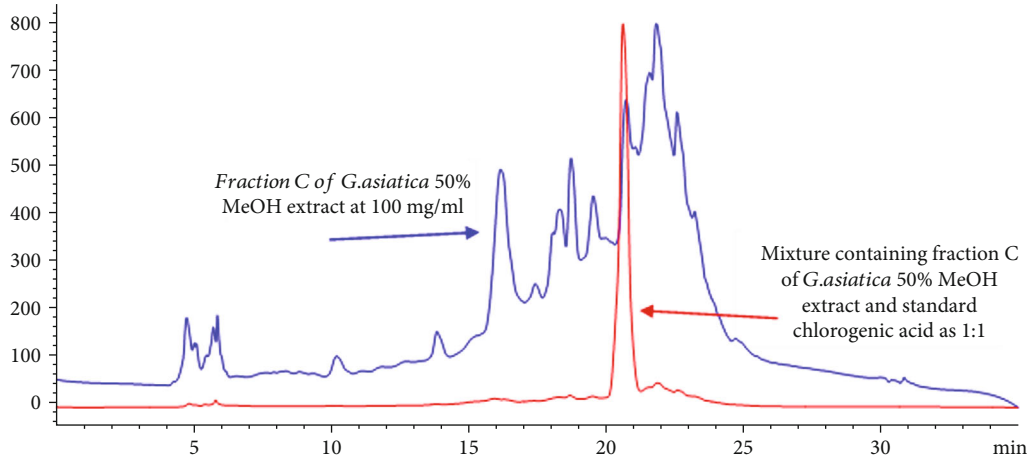

Figure 5: Overlay chromatogram of Fraction C of *G. asiatica* 50% MeOH extract and mixture as 1 : 1.

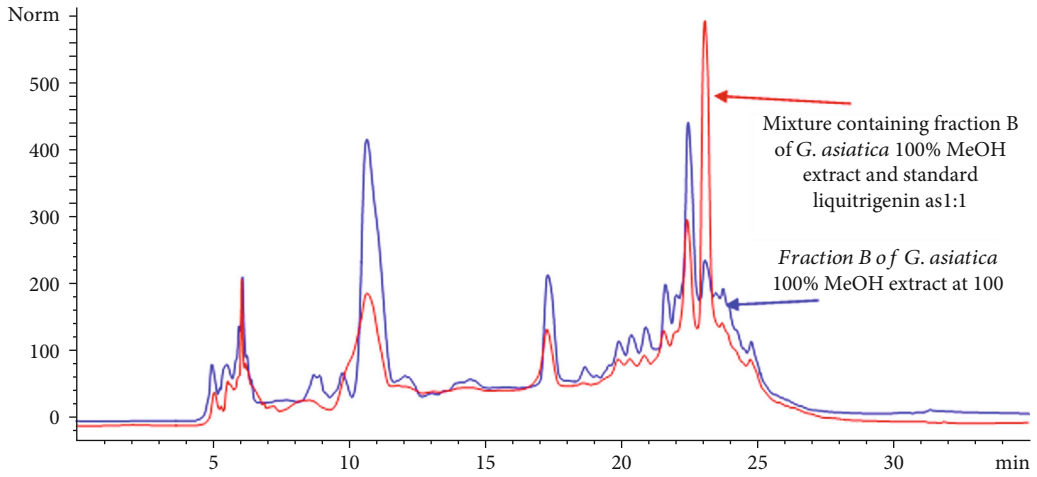

Figure 6: Overlay chromatogram of Fraction B of *G. asiatica* 100% MeOH extract and mixture as 1 : 1.

Table 5: Precision validation of analytical method of three phenolic acids and four flavonoids from *G. asiatica* different fruit fractions.

Marker substance	Theoretical concentration (μg/mL)	Intraday precision ($n = 3$) Measured concentration (μg/mL)	CV (%)	Interday precision ($n = 9$) Measured concentration (μg/mL)	CV (%
Catechin	400	403.6 ± 0.20	0.04	401.4 ± 0.79	0.19
Chlorogenic acid	230	231 ± 1.75	0.75	230.9 ± 0.24	0.10
Caffeic acid	100	102.4 ± 0.28	0.27	100.9 ± 0.31	0.30
Morin	500	498.4 ± 0.57	0.11	499.2 ± 1.13	0.22
Myricetin	150	151.8 ± 1.15	0.75	150.9 ± 0.83	0.55
Quercetin	20	19.6 ± 0.22	1.1	19.9 ± 0.01	0.05
Liquiritigenin	200	201 ± 0.74	0.36	200.9 ± 0.39	0.19

[a]Values shared are mean ± SD of triplicates [b]Values shared are mean ± SD of triplicates for 3 days. Percent coefficient of variation (% CV); (SD/Mean) × 100.

mass spectra of external standards with HPLC chromatogram peaks of *G. asiatica* fractions. Firstly, a visible separation effect was obtained for catechin, chlorogenic acid, caffeic acid, and morin eluted at 19.3, 20.7, 21.8, and 22.6 minutes, respectively, from standard solution and from GAHAF5 (Figure 3). Likewise, it has been apparent from Figure 4 that well-distinguished peaks of myricetin, quercetin, and liquiritigenin were established and eluted at 24.1 minutes (myricetin), 25.0 minutes (quercetin), and 23.2 minutes (liquiritigenin). Chromatograms of GAMF3 also

showed visible peaks of quercetin, liquiritigenin, and myricetin at similar retention times.

3.5.2. Quantification Parameters. The current method was validated for linearity performance using calibration curves. Seven calibration curves in triplicate were established. The calibration curves of catechin, chlorogenic acid, caffeic acid, morin, myricetin, quercetin, and liquiritigenin were linear between 46.8 and 1500, 7.8 and 500, 7.8 and 500, 46.8 and 1500, 12.5 and 200, 1.6 and 25, and 3.9 and 250 μg/mL with a regression coefficient (r^2) between 0.9985 and 0.9995. Regression data, LODs, and LOQs for all seven standard substances are given in Table 3.

3.5.3. Accuracy Validation. Bioactive fractions of *G. asiatica*, i.e., GAHAF5 and GAMF3, were spiked with standards of varying concentrations, i.e., 200, 400, and 500 μg/mL. Recovery rates of the compounds listed in Table 4 were within the range of 98.1 ± 1.01-104.1 ± 1.53% with a percent variation coefficient between 1.02 and 3.65% (Table 4). HPLC overlay chromatograms were also developed to identify the percentage recovery of the bioactive compounds of *G. asiatica.* Figures 5 and 6 represent overlay chromatograms of *G. asiatica* 50% MeOH extract, Fraction *C* with standard chlorogenic acid (1 : 1), and 100% MeOH derived Fraction B with standard liquiritigenin (1 : 1), respectively.

3.5.4. Precision Validation. Instrumental precision was determined by replicate analysis of external standards. The results of intraday and interday analysis of all seven standard compounds showed a high precision with coefficient of variation below 2%, which demonstrates the good precision of our analytical investigation (Table 5).

3.5.5. Quantification Analysis. In this study, the chromatogram peak heights of both fractions (GAHAF5, GAMF3) were compared with those of external standards. The peak height with similar retention times indicates the presence of the respective compounds whose levels were quantified using calibration curves obtained with the corresponding external standards. The detected levels of catechin, chlorogenic acid, caffeic acid, and morin in GAHAF5 were 1230, 491, 957, and 130 μg/g, respectively. Likewise, the levels of liquiritigenin, quercetin, and myricetin in GAMF3 were 217, 591, and 24 μg/g. Earlier, quercetin was detected as 2.4 ng/μl and 4.28 ng/μl in *G. asiatica* callus and leaf extracts, respectively [60]. More recently, LC-QToF-MS analysis of *G. asiatica* extracts tentatively identified and provided relative abundance of some bioactive compounds like quercetin, myricetin, umbelliferone, isovitexin, petunidin, kaempferol, and morin as 0.44, 4.87, 0.10, 0.33, 0.60, 0.87, and 4.25 μg/g, respectively [61].

4. Conclusion

Bioassay-guided fractionation of *G. asiatica* fruit extracts proved to be promising as a means to obtain bioactive fractions bearing significant *in vitro* antioxidant, antiinflammatory, and *in vitro* anticancer activity. Bioactive compound characterization data suggest that chlorogenic acid, caffeic acid, gallic acid, and morin represent the key components of GAHAF5 responsible for the cytotoxic effect on breast, lung, and laryngeal cancer cells. Bioactive fractions from the *G. asiatica* fruit extracts as recovered in this study are potential drug leads and warrant additional testing and determination of therapeutic index *in vivo*.

Abbreviations

RP-HPLC:	Reverse phase high performance liquid chromatography
ESI-MS/MS:	Electrospray ionization mass spectrometry
MCF-7:	Breast cancer cell line
HEp-2:	Laryngeal cancer cells
NCI-H522:	Lung cancer cell line
DPPH:	2,2-diphenyl-1-picrylhydrazyl
FRAP:	Ferric reducing antioxidant power
H_2O_2:	Hydrogen peroxide
MTT:	Methyl thiazolyl tetrazolium
GAMF:	*Grewia asiatica* methanolic fraction
GAHAF5:	*Grewia asiatica* hydroalcoholic fraction
100% MeOH:	100% methanolic extract
50/50 H_2O:	MeOH 50% hydromethanolic extract
TFA:	Triflouroacetic acid
LOD:	Limit of detection
LOQ:	Limit of quantification
r^2:	Regression coefficient
Rt min:	Retention time in minutes.

Data Availability

The data supporting the conclusion of this study are included and are available within the article.

Disclosure

The data presented in this manuscript is a part of the doctoral thesis of Mr. Muhammad Qamar, Institute of Food Science & Nutrition, Bahauddin Zakariya University, Multan, Pakistan.

Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Materials

Supplementary Figures 1 and 2: raw data of ES-MS/MS analysis of GAHAF5 and GAMF3 fractions showing spectras of known and unknown compounds. Supplementary Table 1: qualitative screening of *G. asiatica* fruit extracts showing presence of secondary plant metabolites. *(Supplementary Materials)*

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Review Article

Plants in Anticancer Drug Discovery: From Molecular Mechanism to Chemoprevention

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Cancer is one of the primary causes of mortality globally, and the discovery of new anticancer drugs is the most important need in recent times. Natural products have been recognized as effective in fight against various diseases including cancer for over 50 years. Plants and microbes are the primary and potential sources of natural compounds to fight against cancer. Moreover, researches in the field of plant-based natural compounds have moved towards advanced and molecular level understandings from the last few decades, leading to the development of potent anticancer agents. Also, plants have been accepted as abundant and prosperous sources for the development of novel therapeutic agents for the management and prevention of different cancer types. The high toxicity of some cancer chemotherapy drugs, as well as their unfavorable side effects and drugs resistance, drives up the demand for natural compounds as new anticancer drugs. In this detailed evidence-based mechanistic review, facts and information about various medicinal plants, their bioactive compounds with their potent anticancer activities against different cancers have been gathered, with further approach to represent the molecular mechanism behind the anticancer activity of these plants. This review will be beneficial for investigators/scientists globally involved in the development of natural, safe, effective, and economical therapeutic agents/drugs against various cancers. This might be an important contribution in the field of drug discovery, where drugs can be used alone or in combination to increase the efficacy of newly synthesized drugs.

1. Introduction

Irrespective of advancements in oncology, cancer is yet one of the life-threatening diseases throughout the world. In 2020, approximately 19.3 million new cancer cases were reported globally, resulting in nearly 10 million deaths [1]. Despite being the localize nature of cancer, it can spread throughout the body and various organs via process of metastasis including invasion and migration [2]. From the primary location, metastasis spreads cancer cells to different locations through lymphatic or blood circulation in the body [3]. Metastasis is an intricate process comprising of multiple steps which begin with detachment, amassing, and motility of cancerous cells, resulted in attachment to endothelial cells followed with growth of cancer cells at specific sites [3, 4]. Metastasis is the leading cause for cancer-associated death, due to resistance to diverse cytotoxic agents as well as apoptosis. This is the reason of high mortality and morbidity in patients with metastatic cancer, where present chemotherapy drugs are unsuccessful in effectively eradicating the cancer cells without damaging healthy cells [5].

Medicinal plants are a gift from nature to humans, assisting them in their quest for better health. Since prehistoric times, natural products have been recognized and used by mankind as the chief source of remedial drugs. They are still the source of excellent and potent bioactive compounds, which can directly be used as medicines [6–8]. Several plants have been found to have various kinds of biological activities, like to cure wound, skin diseases, menstrual disorders, after childbirth, fever, headache, exhalation channel infection, urethral infection, rheumatism, and cancer [9, 10]. According to current data, the plant kingdom contains nearly 250,000 plants species, of which only about 10% of medicinal plants have been studied/discovered so far for the treatment of many diseases [11, 12]. Therefore, phytochemical properties of different plants and their derived bioactive analogues occur in assorted portions of plants; like as flower, seed, stem, bark, fruit, leaf, and embryo (Figure 1) [11, 13]. Moreover, all these medicinal plants are eminent to yield numerous bioactive metabolites with various pharmacological attributes together with antidiabetic, antiosteoporotic, antimicrobial, hepatoprotective, anti-inflammatory, antimalarial, antiageing, immunomodulator, antioxidant, antihypertension, anticancer, and others (Table 1) [14–17]. Many have been shown to have anticancer properties; inhibiting cancer cell-related activating proteins, signaling pathways, and enzymes such as topoisomerase enzyme, cyclooxigenase, MMP, MAPK/ERK, TNK, Akt, cytokines, Bcl-2, PI3K, CDK4 kinases, CDK2, CDC2, mechanistic target of rapamycin (mTOR), or by activating DNA repair mechanism [11, 18, 19]. Therefore, choosing these very specific plants, which are described in detail in this review along with their mode of action and possible mechanism, and could potentially be used in the management and prevention of cancer was the primary motivation. This review is intended to open up new avenues for the various kind of treatment/therapy of cancer, focusing on certain important medicinal plants and their important bioactive compounds that have been presented to target cancer cell-activating proteins, signaling pathways, and enzymes involved in cancer progression.

2. Major Medicinal Plants with Anticancer Activities and Possible Mechanism

2.1. Taxus chinensis (Pilger) Rehd (T. chinensis). The most common herbal medicinal component identified from *T. chinensis* is paclitaxel (PTX), a well-known first-line chemotherapy treatment/therapy for cancer disease such as ovarian and breast cancer [20]. Low bioavailability makes oral dose inoculation/administration of pure PTX as a possible anticancer drug difficult. Paclitaxel, a taxane-type diterpenoid, was discovered from the barks of *T. chinensis* and is currently this bioactive compound used as a broad-spectrum chemotherapeutic treatment/therapy against various cancer categories [21]. Numerous findings have demonstrated that PTX can cause apoptosis and cell cycle arrest by blocking microtubule depolymerization and boosting microtubule polymerization, and PTX's unusual structure and anticancer potency have piqued researchers' attention around the world [22]. The taxane extract from *T. chinensis* enhances paclitaxel bioavailability through pharmacokinetic synergy [23]. The current approach to taxol production is natural source obtained from the bark of *T. brevifolia*, which is considered as a most rich source; nonetheless, based on this method, the yield of taxol ranged from 0.001 to 0.05 percent, meaning that 8–10 kg of plant bark from 4–5 plants is required to produce one gram of pure taxol [24]. However, this supply faces issues due to its limited availability and increased vulnerability to unpredicted changes in biological and environmental conditions. Camptothecin and taxol were the first compounds with high antiproliferative activity to be studied after the National Cancer Institute program of research for novel anticancer chemicals began in 1960 [25]. NCI clinical trials have looked at taxol in the following stages of different cancers: Phase I (childhood leukemia, lymphoma, epithelial, liver, breast, and ovarian), Phase II (melanoma, head and neck, esophageal, small cell lung, colon, renal, and prostate), and Phase III (melanoma, esophageal, and melanoma) (ovarian epithelial and metastatic breast) [25–28]. Taxol was licensed by the FDA in 1992/94 as an effective treatment for breast and ovarian cancer, respectively [28]. As a result, this bioactive compound (taxol) was regarded as one of the most significant contributions to the sector of chemotherapeutics in the late twentieth century [28].

Taxol is a highly effective anticancer treatment that works against a variety of malignancies, which include ovarian, lung, breast, head carcinomas, and AIDS-related Karposi's carcinoma [29]. Proliferation of cancer cells occurred due to abnormal cell growth and division, which causes a surge in the number of cells [29]. Tubulin is a globular protein that is located in the cytoskeleton of eukaryotic cells and plays a vital part in cancer cell mitosis [30]. During mitotic division, the dynamics of microtubules, which involves depolymerization (shrinking) and polymerization (growing), is critical for chromosome separation (Figure 2). Two various kinds of tubulins polymerize together to generate heterodimer microtubules, which are assembled to form protofilaments. Microtubules in live cells have been found to have 13 protofilaments arranged parallel to the microtubule axis [30–32]. The positive charged end of a microtubule

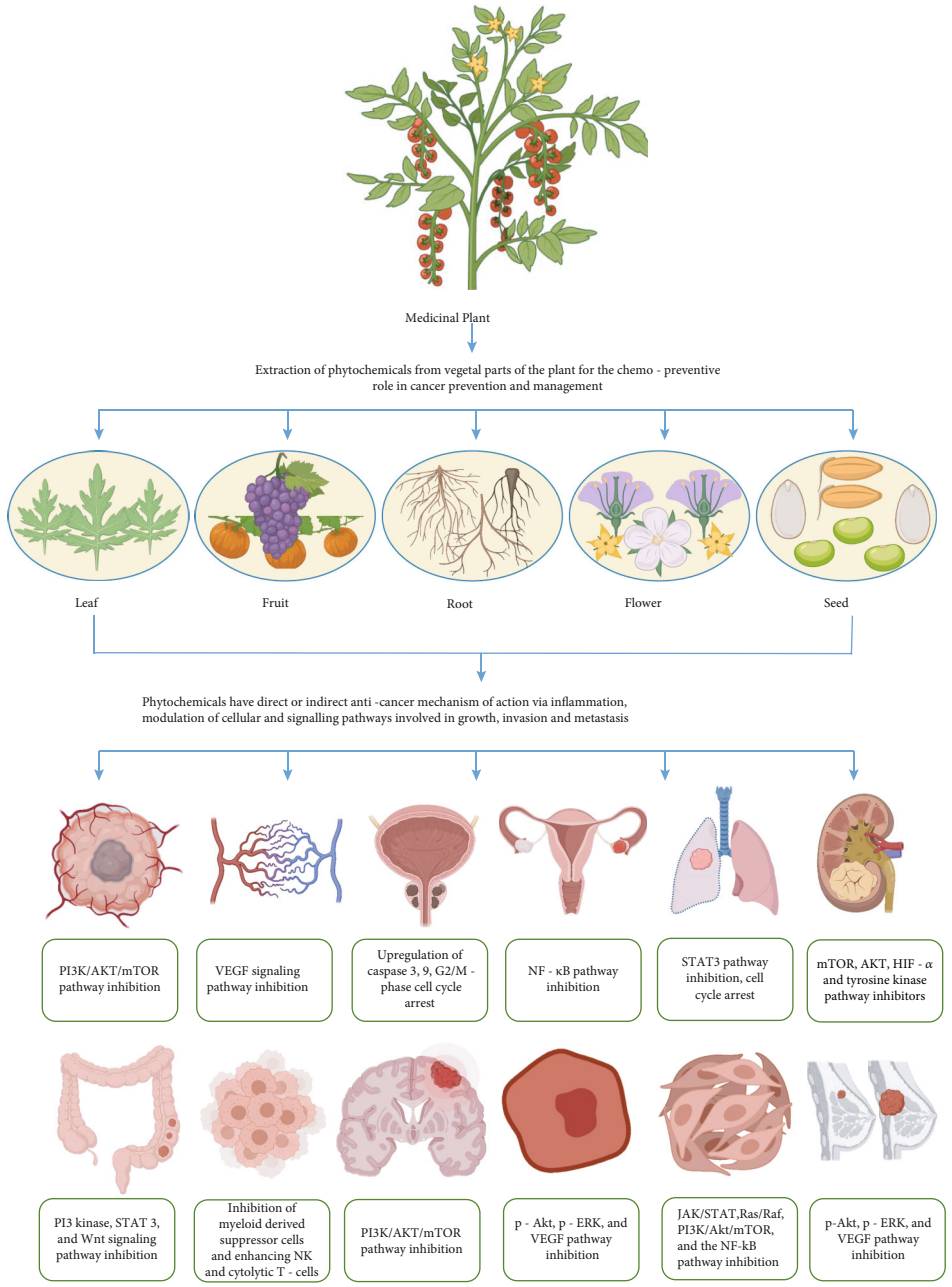


Figure 1: Pictorial depiction of vegetative parts of medicinal plant involved in therapeutic effects against various types of cancers and their target pathways.

(+tubulin) connects to the kinetochore of chromosomes, while the negative charged end (-tubulin) binds to the spindle pole. When a drug attaches/binds to tubulin, it changes the way microtubules assemble [30, 32]. Taxol is an antiproliferative medication that works in a unique way against cancer cells. It interacts with tubulin through an amino-terminal region of amino acids (that is 31 amino acid), preventing microtubule depolymerization and halting the cell cycle [31]. Microtubules organized to have 12 proto filaments rather than 13 proto filaments in the presence of taxol [32].

2.2. Curcuma longa L. (C. longa). Turmeric or *C. longa*is an aromatic, nutraceutical plant. The root, a vegetal product of this plant, has been extensively utilized in Indian traditional medicine (Ayurveda) for various maladies, including parasite infection (local administration), wounds, acne, urinary tract disease, common cold, and liver disease, under various pharmaceutical formulations (systemic administration) [33, 34]. Numerous experimental studies on turmeric's therapeutic activity have revealed a wide -range of pharmacological properties, such as anti-HIV, antibacterial, antioxidant, anti-angiogenic, anti-inflammatory, proapoptotic, immunomodulatory, analgesic properties with applications in Alzheimer's disease, diabetes, and arthritis [35, 36]. The main active ingredient responsible for the pharmacodynamics activity is curcumin, a polyphenol, which has displayed

Table 1: List of plants and their potent bioactive phytocompounds for the possible therapeutic use in prevention and management of various cancers.

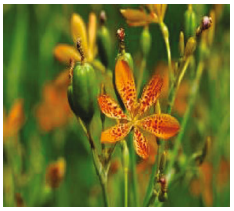
Vegetal part and botanical name	Picture	Bioactive phytocompounds	Biological functions	Therapeutic effect against various cancers	References
Taxus chinensis (Pilger) Rehd (bark)		Paclitaxel (taxol)	Anticancer, antioxidant, and antiageing	Breast, liver, ovarian, colon, lung and esophageal cancer, lymphoma, childhood leukemia, melanoma	[20–22, 25, 26, 150]
Curcuma longa Linnaeus (rhizome)		Curcumin, demethoxycurcumin, bisdemethoxycurcumin, germacrone, furanodienone, zederone, and ar-turmerone	Anticancer, antiangiogenic, antioxidant, anti-inflammatory, anti-HIV, antibacterial, and immunomodulatory	Colon, cervical, lung, thymic, brain, pancreatic, breast, bone cancers, and liver cancer	[34–37, 151]
Zingiber officinale roscoe (rhizome)		Phenolic and terpene	Anticancer, anti-inflammatory, antioxidant, immunomodulatory, antiangiogenic, and antibacterial	Colon cancer, ovarian cancer, and breast cancer	[41–43, 46, 152]
Camptotheca acuminata Decne (leaf, flower, stem, fruit, root)		Alkaloids, flavonoids, and glycosides	Anticancer, antivirus, antipsoriatic, antifungal, anti-inflammation, antibacterial, and antiparasitic	Lung and ovarian cancer	[48–50, 153]
Vinca rosea L. (*Catharanthus roseus* (L.) G. Don) (leaf)		Vinca alkaloids: vindesine, vincristine, vinorelbine, and vinblastine	Anticancer, antioxidant, anti-inflammatory	Lung cancer, breast cancer, Hodgkin's lymphoma, leukemia, Kaposi sarcoma, Ewing sarcoma, follicular lymphoma, retinoblastoma, ovarian germ cell tumor, acute lymphoblastic leukemia rhabdomyosarcoma, testicular germ cell tumor	[54–56, 154]
Belamcanda chinensis L. DC. (root)		Flavonoids, terpenoids, organic acids, and quinones	Antitumor, antioxidant, antibacterial, antidiabetic, estrogen-like, hepatoprotective, and anti-inflammatory	Breast cancer, liver cancer, prostrate carcinoma, gastric carcinoma, T-cell leukemia	[61–63, 65, 155]

Table 1: Continued.

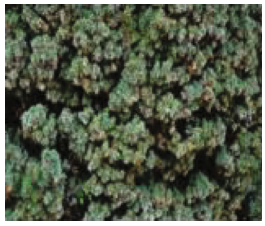
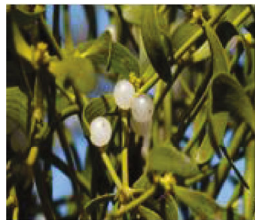
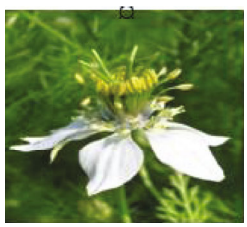
Vegetal part and botanical name	Picture	Bioactive phytocompounds	Biological functions	Therapeutic effect against various cancers	References
Cryptolepis sanguinolenta (Lindl.) Schltr. (leaf and root)		Alkaloids, flavones, and tannin	Anticancer, antibacterial, antiparasitic, anti-inflammatory, antifungal, antidiabetic, and antioxidant	Lung cancer	[66, 67, 156]
Garcinia hanburryi hook (fruit, leaf, and seed)		Polyphenols, benzophenones, xanthones, and bioflavonoids	Anticancer, anti-inflammatory, antioxidant, antitumor, antifungal, antiulcer, antibacterial, antiviral.	Breast cancer, lung cancer, hepatocellular carcinoma, prostate cancer, and gastric carcinoma.	[74–77, 157]
Psoralea corylifolia L. (Buguchi) (whole plant)		Psoralidin, meroterpenes, coumarins, and flavonoids	Anticancer, anti-inflammatory, antibacterial, antidepressant, antioxidant, and antipsoriatic.	Breast, prostate, and lung cancer	[81, 82, 84, 158]
Cimicifuga foetida L. (rhizome)		Phenylpropanoids, lignans, cycloartane triterpenoids, chromones, amides	Antitumor, anti-inflammatory, antiviral, antimenopause, analgesic, antiosteoporosis, and antipyretic	Breast, prostate, and liver cancer	[88, 89, 93, 159]
Taxus baccata L. (leaf and bark)		Paclitaxel (taxol), taxusin, baccatin, taxoids viz., baccatin, taxine, lignans, phenols steroids, flavonoids	Anticancer, antimalarial, antirheumatic, abortifacient, anti-inflammatory antinociceptive, and septic shock	Kaposi's sarcoma, breast, ovarian, and lung cancer	[94, 96, 97, 160]
Viscum Album L. (stem, leaf, and fruit)		Flavonoids, phenylpropanoids, alkaloids, proteins, triterpenes, steroids, lipophilic molecules, viscumneoside XII, XIII, XIV, lectins, and conjugated acetylene	Anticancer, antidiabetic, antioxidant, anti-inflammatory, sedative, antihypertensive, and hepatoprotective	Breast cancer and gynecological cancer	[101, 102, 109, 111, 161]

Table 1: Continued.

Vegetal part and botanical name	Picture	Bioactive phytocompounds	Biological functions	Therapeutic effect against various cancers	References
Gardenia jasminoides J. Ellis (stem, bark, and fruit)		Geniposide, crocin, genipin, gardenoside, and iridiod	Anticancer, antidepression activity, antioxidant, hypoglycemic effect, antidiabetic, anti-inflammatory, improvement in the quality of sleep, antigastritis, antiarthritis, antihyperlipidemia	Brain tumor, oral cancer, liver cancer	[113, 114, 116, 162, 163]
Colchicum autumnale L. (bulb, flowers, and leave)		Colchicine	Anticancer, anti-inflammatory, and antiproliferative	Solid tumor, leukemia	[118, 119, 121, 164]
Salvia prionitis Hance (root)		Diterpenoid quinone, salvicine	Anticancer, antitumor, antimicrobial	Lung cancer and solid tumor	[122, 124, 127]
Raphanus sativus L. (root, stem, leaf)		Flavanoid, glucosinolates, folic acid, flavonoids, polyphenolics, dietary fiber, vitamin A and C	Anticancer, antimicrobial, and anti-inflammatory	Colon cancer and breast cancer	[128–131, 165]
Tinospora cordifolia (Willd.) Miers (bark, leaf, flower, and stem)		Polysaccharides, aliphatic compounds, phenolics, sesquiterpenoid, steroids, diterpenoid lactones, alkaloids, glycosides	Anticancer, antineoplastic, antioxidant, hepatoprotective, hypolipidemic, antiperiodic, antispasmodic, anti-inflammatory, antimicrobial, antiosteoporotic, antidiabetic, antiarthritic, antiallergic, and immunologic	Breast cancer and tumor	[132–137, 166]
Nigella sativa L. (seed)		Thymoquinone, dithymoquinone, and dihydrothymoquinone	Anti-inflammatory, anticancer, antioxidant, antibacterial, antiangiogenic, antidiabetic, and organ-protective	Breast cancer and tumor	[138–142, 167]

potent anticancer effects against numerous cancers [36]. This includes bone, breast, pancreatic, colon, lung, cervical, liver, and thymic (Figure 2) [37, 38]. Recent research strongly supports polyphenols' significance in the prevention of degenerative diseases, cancer, and cardiovascular diseases [38]. Natural polyphenols can occur in a various range of foods, including cereals, chocolates, vegetables, fruits, olive oil, and beverages like wine and tea. Curcuma aqueous extract has been shown to induce apoptosis in human colon cancer LS-174-T cells. Ozaki et al. (2000) revealed the role of curcumin in inducing apoptosis in rabbit osteoclasts as well as inhibiting bone resorption [38, 39]. Curcumin's proapoptotic effect in leukaemic Jurkat cells, COLO 205 cells, human lung carcinoma A549 cells, murine myelomonocytic leukemia WEHI-3 cells, human nasopharyngeal carcinoma cells, and NPC-TW 076 has previously been documented [38, 40]. Furthermore, curcumin is a proapoptotic agent that fights against various lymphoma cells in human [40].

2.3. Zingiber officinale Roscoe (Z. officinale). Z. officinale (Ginger, Zingiberaceae) is a rhizomatous perennial plant used to treat digestive disorders such as dyspepsia, nausea, vomiting, gastritis, diarrhea, asthma, common cold disorders, nervous diseases, inflammation, hepatotoxicity, diabetes, migraine, hypercholesterolemia, helminthiasis, and schistosomiasis [41–43]. Since prehistoric times, ginger has been widely utilized as a spice for culinary and medicinal purposes throughout the world. Ginger has been known to be anticarcinogenic through numerous routes and to have chemo-preventive activity in colon cancer [43]. Human colorectal cancer cells were also suppressed by gingerol (phytochemical found in ginger). Efficacy of ginger has been tested on developed tumor in mice in one study and showed potential reduction in tumor size [44]. In mice, the effects of continuous therapy with ginger rhizome hot water extract on spontaneous mammary cancer were studied. The formation of mammary tumors was dramatically prevented in the mice given free access to ginger extract (0.125 percent) in drinking water [44–46]. Gingerol also induces auto phagocytosis and apoptosis, which destroys ovarian cancer cells (self-digestion). The presence of a proinflammatory state is hypothesized to participate in the enlargement of ovarian cancer. A number of critical inflammatory markers (interleukin-8, vascular endothelial growth factor, and prostaglandin E2) found to be reduced in ovarian cancer cells with treatment of ginger [46]. The antiproliferative and antioxidant activities of the methanol extract of cannibal rhizome on human cervical HeLa cancer cells and human breast cancer MDA-MB-231 cancer cells were also studied [46].

2.4. Camptotheca acuminata Decne (C. acuminata). This medicinal plant belong to Nyssaceae family and basically was found in Tibet and southern China [47]. *C. acuminata* is well know for broad-range of biological activities such as anticancer, antivirus, antipsoriatic, antifungal, anti-inflammation, antibacterial, and antiparasitic [48–50]. Furthermore, *C. acuminata* contains various natural active compounds, like alkaloids, flavonoids, and glycosides [50]. It contains alkaloid camptothecin (CPT) and betulinic acid (BA), which can be isolated from stem, bark, and fruit of *C. acuminata* plant [51]. The anticancer mechanism of action of CPT is by inhibiting topoisomerase 1. CPT binds to a complex, which contain deoxyribose nucleic acid (DNA) and topoisomerase I, hence, inhibiting the reassembly of the DNA strands of a single chain [51, 52]. When the CPT is combined with the DNA–topoisomerase complex, the bonds at the nick sites are not restored, and the double DNA chain structure is damaged [52]. The DNA damage in tumor cells treated with CPT and its derivatives is most likely caused by double-strand breaks introduced by the replication process [25, 52]. Therefore, CPT also has been engaged for the treatment of ovarian and lung cancer [53]. On the other hand, BA is involved in treatment of cancer, HIV, and bacterial diseases.

2.5. Vinca rosea L. (Catharanthus roseus (L.) G. Don). This medicinal and ornamental plant is a member of Apocynaceae family and commonly known as pink periwinkle or Madagascar periwinkle [54]. *V. rosea* is rich in alkaloids with wide-ranging biological activities and use in treatment of several types of cancers, such as lung, breast, leukemia, acute lymphoblastic leukemia, testicular germ cell tumor, Kaposi sarcoma, Hodgkin's lymphoma follicular lymphoma, ovarian germ cell tumor, retinoblastoma, Ewing sarcoma, and rhabdomyosarcoma [54, 55]. Furthermore, *V. rosea* contains Vinca alkaloids: that are vindesine, vincristine, vinorelbine, and vinblastine [56, 57]. All these Vinca alkaloids were the first natural compounds to go into clinical trial against several cancers and have been endorsed and licensed by Food and Drug Administration (FDA) [58]. It has been known that usage of these alkaloids in low doses interferes with the microtubular activity, while at high doses, causing cell cycle arrest and apoptosis [58, 59]. These alkaloids are currently in use for the treatment of various cancers.

*2.6. Belamcanda chinensis L. (B. chinensis). B. chinensis*is a traditional Chinese medicinal plant, belongs to the Iridaceae family and commonly found in northeast Asia region [60, 61]. The biological activity of this plant include antitumor, antioxidant, antidiabetic, estrogen-like, hepatoprotective, antibacterial, and anti-inflammatory [61]. It contains several bioactive compounds like flavonoids, terpenoids, organic acids, and quinones [62]. The flavonoids and terpenoids are mostly used in the treatment of cancer [63]. Terpenoids (pentacyclic triterpenoids) are also one class of natural compounds introduced in clinical trials [63]. However, ursolic acid, betulone, and betulonic acid are isolated from root of *B. chinensis* medicinal plant and mostly all these compounds are utilized in treatment/therapy of numerous type of cancers, such as solid tumor, breast cancer, liver cancer, prostrate carcinoma, gastric carcinoma, and T-cell leukemia [64, 65]. However, against different types of cancer cells, betulonic acid has shown most significant antitumor activity at 20 μmol/L: for example, prostatic cancer cell line PC3 (rate of inhibition = 52%), breast cancer cell line MCF-7 (rate of inhibition = 56%), and human gastric cancer cell line MGC-803 (rate of inhibition = 68%) [63]. Furthermore, the ability of betulonic acid to follow apoptotic pathways

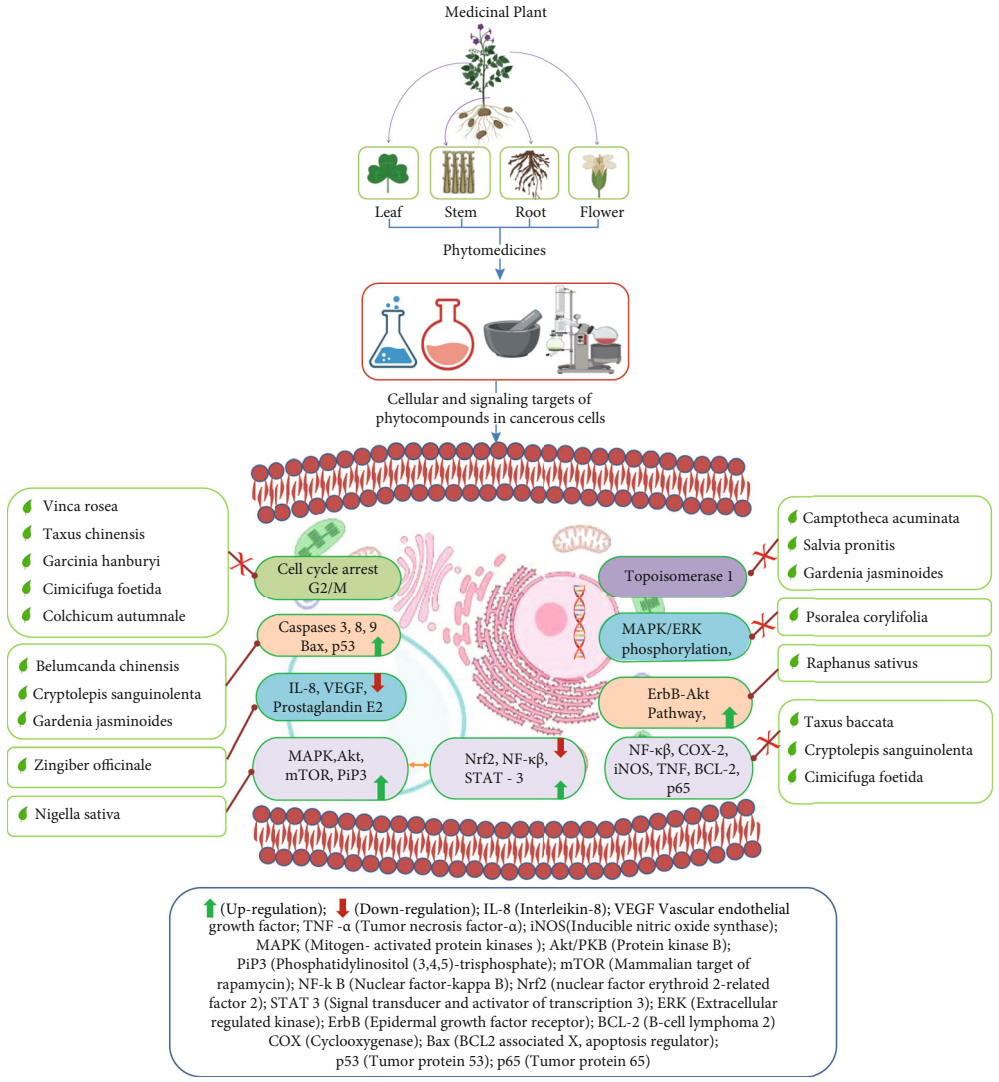


Figure 2: Illustrative representation of potential plants targeting specific cellular and signaling pathways depicting molecular mechanism of their possible anticancer activity.

through the mitochondrial signaling cascade, which implies the expression of caspases 3 and 9, as well as the proteins p53 and Bax, is one of its antitumor properties [63]. Therefore, this is most promising agent which can be used for the cancer chemoprevention or management.

2.7. Cryptolepis sanguinolenta (Lindl.) Schlechter (C. sanguinolenta). C. sanguinolenta is an African scrambling thin-stemmed shrub. It has traditionally been used in West Africa to treat the diarrhea, malaria, and respiratory problems [66]. Furthermore, different synthetic derivatives of specific alkaloidal isolates from *C. sanguinolenta* have also been studied for the anticancer action. *C. sanguinolenta* has been mostly linked to its principal alkaloid, cryptolepine [66, 67], which has demonstrated to block the NF-κB activity in a variety of cells [68]. Along with that, in human lung adenocarcinoma A549 cells, cryptolepine stimulates cell cycle arrest and apoptosis [66, 68]. Cytotoxic and anti-inflammatory effects are mediated by interfering with NF-κB activity, which results in reduction of inflammatory as well as antiapoptotic genes such iNOS, TNF-α, COX-2, and Bcl-2 [68]. The proapoptotic genes p53, p21, Bax, caspase, and cytochrome c are all upregulated when NF-κB is inhibited [69].

Olajide et al. 2013 studied that apoptotic-inducing impact of cryptolepine is mediated through the NF-κB signaling pathway during different study [69]. It was observed that, after 24 hours of therapy, cryptolepine decreased A549 cell growth dose-dependently with upregulation of caspase-3 [69]. Cryptolepine reduced TNF-induced IB phosphorylation and NF-κBp65 nuclear translocation, according to protein analysis [67, 69]. Moreover, significant DNA damage is caused by cryptolepine by blocking the topoisomerase I and II activity [68, 70]. This damage led in increased phosphorylation of BRCA1, Chk1/Chk2, H2AX, and ATM/ATR, as well as p53 signaling cascade activation, which included increased protein expression of the cyclin-dependent kinases p21 and p16 [71]. In human

nonmelanoma skin cancer cells, these cryptolepine-induced alterations resulted in a considerable reduction in cell viability and colony formation, as well as an expend in apoptotic cell death [71].

2.8. Garcinia hanburyi Hook (G. hanburyi). Garcinia is a Clusiaceae genus found in Africa, New Caledonia, Southeast Asia, Brazil, and Polynesia [72]. Garcinia plants contain a wide range of biologically active metabolites that have gotten a lot of interest in recent decades because of the chemical compositions of their extracts, which contain chemicals that have been demonstrated to have positive effects in a variety of ailments [72]. There are several species found, but the following five are the best studied and have numerous therapeutic properties: *G. mangstana*, *G. pedunculata*, *G. gardneriana*, *G. brasiliensis*, and *G. cambogia*. These species are known to have potent anti-inflammatory effects; such as pain, wounds, treatment for skin diseases and infections, with analgesic, antioxidant, antitumor, antifungal, anticancer, and anti-inflammatory properties [73, 74]. They are a rich and natural source of biologically active compounds with various other therapeutic properties Moreover, they also contain amine, with antiulcer, antibacterial, antiviral, vasodilator, hypolipidemic, and hepatoprotective properties [74]. Moreover, *G. hanburyi* has anticancer effects in different kinds of human cancer cells, such as gastric carcinoma, hepatocellular carcinoma, breast cancer, prostate cancer, and lung cancer [75].

Garcinia is rich in polyisoprenylated benzene derivatives (polyphenols, benzophenones, xanthones, and bioflavonoids) [76]. Garcinia-derived biflavonoids have also been studied for a variety of functions, including chemoprevention [77]. Kolaviron (extract from the seed of Gracinia) is believed to have the capability to eliminate free radicals, inhibit stress-related proteins and interfere with the DNA binding activity of certain transcription factors. Thus, it helps as anticancer agent [77, 78]. However, forbesione, a caged xanthone derived from *G. hanburyi*, has been shown in human CCA cell lines to reduce growth and cause apoptosis [77]. In addition, it was found that the expression of p27 and p21 was increased, which may describe why proliferating bile duct cell markers and cell nuclear antigen in forbesone-treated Ham-1 cells *in vitro* and in forbesone-treated hamster tumor tissues Cytokeratin 19 are reduced. In addition, forbesione promotes apoptosis through a variety of mechanisms [79]. Increased expression of Fas, Fas-related death domains, and activated caspase-3, as well as decreased expression of procaspase-3 and 8, activates the death receptor pathway [79, 80]. Increased expression of activated caspase-9, B-cell lymphoma- (Bcl-) 2-like protein 4 and B-inhibitors, expression of Bcl-2, survivin, procaspase-9, and nuclear factor-B decreased p65, driving mitochondria way [80]. Lower expression of procaspase-12 and higher expression of activated caspase-12 stimulated the endoplasmic reticulum pathway. In forbesione-treated hamsters, no adverse effects or toxicity were identified. As a result, forbesione is observed as a promising cancer treatment candidate that warrants additional research [79, 80].

2.9. Psoralea corylifolia L. (Buguchi) (P. corylifolia). This medicinal plant is member of Leguminosae family and was used in ancient times for the treatment of several ailments and is originally distributed in Asian regions [81]. It is known forwide-range of biological activtiessuch as anticancer properties, anti-inflammatory, antibacterial, antidepressant, antioxidant, and antipsoriatic [81]. Furthermore, this medicinal plant encompasses numerous bioactive components like as psoralidin, meroterpenes, flavonoids, and coumarins. Furthermore, bioactive compounds psoracoumestan and arylcoumarin from *P. corylifolia* displayed robust anticancer potential by strongly blocking MAPK/ERK kinase phosphorylation enzyme system [82, 83]. Another bioactive compound psoralidin has found to be potentially important in treatment of cancer. Psoralidin is involved in different types of cancers like breast, liver, and others [82, 84]. In a study, psoralidin an estrogen receptors agonist was found to induce pS2 gene activity in MCF7 breast cancer cells with an EC_{50} value of ERE-reporter gene transcription activity of 1.85 μM [82, 85]. While, seed extract of *P. corylifolia*induced apoptosis in the human breast cancer (MCF-7) cells followed by mitochondrial cell death [86]. Another study stated that combination of psoralidin and neobavaisoflavone with TNF-related apoptosis-inducing ligand (TRAIL) has highlighted their anticancer activity through inducing apoptosis in human adenocarcinoma prostate cancer cells [84, 87]. Another study clearly revealed that psoralidin combination with TRAIL influences apoptosis in HeLa cells by upregulating the expression of death receptor [84, 87]. Furthermore, in the same study, it has been showed that psoralidin bioactive compound have anticancer activity against human lung cancer cell by using different extracts of *P. corylifolia* [84, 87].

2.10. Cimicifuga foetida L. (C. foetida). This medicinal plant is a member of Ranunculaceae family and originally was found in Asian region such as China, India, and Tibet [88]. This medicinal plant has been used a traditional Chinese herbal medicine globally since old times and commonly known as Shengma [88]. *C. foetida* is mostly used in the treatment/cure of headache, sore throat, aphtha, uterine, prolapse, archoptosis, spot poison, and nonerupting measles and many other related diseases [88, 89]. *C. foetida* has wide-ranging biological activities comprising like as antitumor, anti-inflammatory, antiviral, antimenopause, analgesic, anti-osteoporosis, and antipyretic [88, 89]. Its potent bioactive compounds are phenylpropanoids, lignans, amides, chromones, cycloartane triterpenoids, and other compounds, which are extracted from the rhizome of *C. foetida* [90]. Therefore, phenolic compounds and triterpenoids have been shown effective anticancer activities in both *in-vitro* and *in-vivo* studies [91, 92]. Furthermore, *C. foetida* is often used as estrogen-based replacement therapy to get rid of menopausal symptom and detoxification. Triterpenoids in *C. foetida* synergistically impedes the proliferation of hepatocellular cells, breast cancer cell line, and prostate cancer by triggering cell cycle G2/M arrest and apoptosis [93]. Moreover, triterpenoid (KHF16) compounds have the ability to induce cell cycle G2/M phase arrest and apoptosis in

different cell lines, as well as potential to block TNFα-induced p65 nuclear translocation, IKKα/β phosphorylation in ERα/PR/HER2 triple-negative breast cancer cells [93]. Hence, bioactive compounds from *C. foetida* have great potential to inhibit several cancers and must undergo for clinical trials.

2.11. Taxus baccata L. (T. baccata). This plant is a member of Taxaceae family and generally known as English yew or European yew. It is widely found in Eastern Asia, Europe, and North America [94, 95]. *T. baccata* is mostly used for ornamental landscaping and inflammatory diseases, due to the occurence of lignin derivatives. *T. baccata* contains large amount of bioactive compounds such as Paclitaxel (taxol), taxusin, baccatin, taxoids viz., baccatin, taxine, lignans, phenols, phenolic glucoside, sugar derivatives, steroids, and flavonoids (3-O-rutinoside quercetin, 3-O-rutinoside myricetin, and quercetin) [96, 97]. *T. baccata* possesses a wide range of biological activities; anticancer, antimalarial, antirheumatic, abortifacient, anti-inflammatory, antinociceptive, and septic shock activities [96, 97]. However, two bioactive compounds of *T. baccata*, lariciresinol and isolariciresinol, have been reported for anticancer activities [98]. Lariciresinol and isolariciresinol are lignans, which were revealed to have effective inhibitory effect on tumor necrosis factor (TNF) *in vitro* [98]. It is known that TNF is one of the main Th1 cytokine released during initial phase of chronic, and acute inflammatory diseases includes rheumatoid arthritis, asthma, and septic shock [99]. Therefore, paclitaxel (taxol) and taxanes isolated from *T. baccata* have been permitted for therapeutics of numerous cancer-related diseases such as lung cancer, Kaposi's sarcoma, ovarian cancer, and breast cancer [28, 96]. Currently, paclitaxel and taxanes are under clinical trial for the treatment of other cancers in combination with other bioactive compounds/chemotherapeutic medications [96]. Furthermore, taxanes have potential to inhibit the mitosis division.

2.12. Viscum album L. (V. album). This plant (*V. album*) is a hemi-parasitic, evergreen shrub plant is a member of Santalaceae family and commonly known as Mistletoe or European mistletoe [100]. It is widely distributed in Europe, southwest and northwest Africa, and central Asia. Several bioactive compounds are known to be present in *V. album* such as flavonoids, phenylpropanoids, alkaloids, proteins, carbohydrates, oligosaccharides, polysaccharides, triterpenes, steroids, lipophilic molecules, viscumneoside XII, XIII, XIV, lectins, and conjugated acetylene compounds [100–102]. Viscotoxins are thionines and are classified as alpha and beta is the major low molecular protein of *V. album*, which is extensively reported [103]. The biological activities of *V. album* have been reported to be anticancer, antidiabetic, antioxidant, anti-inflammatory, sedative, antihypertensive, and hepatoprotective [101]. Furthermore, *V. album* has been used mostly to treat high blood pressure, arthrosis, hemorrhages, arteriosclerosis, diabetes, menstrual disturbances, migraines, epilepsy, endometriosis, asthma bronchiale, and neuralgias in the last 200 years [104, 105]. Several preclinical studies have been reported that *V. album* showed proapoptotic, immunostimulatory, and cytotoxic effects [106]. Furthermore, in mouse/animal models, *V. album* extract has showed direct antitumour activity, while indirect activity showed via initiation of various immune system pathways [106]. The main phytochemicals of *V. album* such as lectins and viscotoxins are described to have anticancer activity [107]. Lectins and viscotoxins play an important role in cancer treatment because of their cytotoxic and apoptotic effects. They are further able to increase cytokine secretion, stimulate immune cells phagocytosis, induce macrophage cytotoxicity, and enhance *in vitro* cytotoxic effects on various cell lines [107, 108]. Efficacy of *V. album* on the activity of natural killer (NK) cells and T-cytotoxic cells was also investigated in another study [108]. Although inhibition of NK cells cytotoxic activity was also discovered in some studies, the majority of them showed an increase in cell concentration and improved function [109, 110]. Whereas, several studies confirmed the stability of tumor, reduction in tumor growth, or reduction in metastases with improved survival rate [108, 109, 111]. Hence, *V. album* is an excellent example of medicinal plant that acts as a link between conventional and alternative medicine.

2.13. Gardenia jasminoides J. Ellis (G. jasminoides). *G. jasminoides* belongs to Rubiaceae family and mostly found in Asian reason such as China, India, Korea, Nepal, Tibet, and Bhutan. It is mostly used in the treatment of hypertension, edema, inflammation, fever, headache, jaundice, and hepatic disorders [112, 113]. *G. jasminoides* possesses several biological activities; anticancer, antidepression, antioxidant, hypoglycemic effect, antidiabetic, anti-inflammatory, improvement in the quality of sleep, antigastritic, antiarthritis, antihyperlipidemia, and also inhibition of retinal damage [113, 114]. *G. jasminoides* is also widely used as a natural yellow dye and as a traditional Chinese medicine since ancient times. *G. jasminoides* contains many bioactive compounds such as geniposide, crocin, genipin, gardenoside, and iridiod [113]. However, chemical components of this medicinal plant have been isolated and characterized as/including volatile compounds, glucosides, triterpenoids, organic acids, and iridoid [114]. Aliphatic acids, esters, alcohols, ketones, aldehydes, and aromatic derivatives are the main volatile compounds found in *G. jasminoides* essential oil [106]. Furthermore, *G. jasminoides* fruit contains iridoid glycosides and crocin as main bioactive compounds with a potential to exhibit antitumor, antioxidant, cytotoxic, and antihyperlipidemic effects [115, 116]. *G. jasminoides* extract can inhibit the activity of topoisomerase 1, which seemingly encourages the formation of supercoiled DNA [116, 117]. Lim et al. (2010) reported that on KB oral cancer cells, the cytotoxic effect of *G. jasminoides* extract dichloromethane fraction increased in a dose-dependent manner [117]. This cytotoxicity was not seen in the normal human epidermal keratinocyte HaCaT cell line, but was found to be effective against oral cancer KB cell line. Furthermore, Lim et al. (2010) demonstrated that the dichloromethane fraction of *G. jasminoides* extract induced apoptosis by increasing the activities of caspase-3, 8, and 9, as well as the cleavage of poly (ADP-ribose) polymerase [117]. Hence, these novel findings

suggest that *G. jasminoides* extract could be a candidate for the development of novel anticancer drug.

2.14. Colchicum autumnal L. (C. autumnale). C. autumnale, commonly known as autumn crocus, is a flowering plant which belongs to the family of Colchicaceae. It is native to Great Britain and Ireland. *C. autumnale* is considered as a toxic plant because of the presence of colchinine, which is found in the bulb like corms of the plant [118]. Colchicine is the main alkaloid of *C. autumnale*. It has narrow therapeutic index and is used effectively as a remedy against gout, Behçet's disease, and familial Mediterranean fever at many places [119]. The pain-relieving, anti-inflammatory, and antiproliferative effects of colchicine are closely linked with its ability to bind with tubulin, which plays an important role in cell division. Colchicine blocks the cell cycle at the G2/M phase inhibiting the action of tubulin, thereby the formation of microtubule severely damages the internal scaffolding of the cells and trigger apoptosis [120]. Owing to its high toxicity, colchicine has not found significant use in cancer treatment; however, it is still used as a lead compound for the generation of potential anticancer drugs [121].

2.15. Salvia pronitis Hance (S. prionitis). S. prionitis is an annual herb, which belongs to the family of Lamiaceae. It is native to the southern part of mainland China. A diterpenoid quinone named as Saprorthoquinone is the main compound isolated from *S. prionitis* and is used as remedy of fever, tonsillitis, pneumonia, and diarrhea [122, 123]. In *in vivo* xenograft models of LAX-83 lung adenocarcinoma cells, A-549 lung, Lewis lung, and S-180 sarcoma, Salvicine, which is a diterpenoid quinone from *S. prionitis*, displayed significant growth inhibitory activity [124, 125]. It also bears cytotoxic effect on multidrug-resistant (MDR) tumor cells [126]. Salvicine exerts its antineoplastic effects and induces apoptosis by acting as a nonintercalative topoisomerase II inhibitor. Additionally, Salvicine increases the level of intracellular H_2O_2 and is also linked with the occurrence of DNS double-strand breaks [127]. Salvicine directly reacts with GSH and results in significant depletion of intracellular GSH. It has been suggested that both GSH-depletion-driven H_2O_2 generation and Topo II inhibition are critical for Salvicine-mediated DNA double-strand breaks and apoptosis [127].

2.16. Raphanus sativus L. (R. sativus). R. sativus is an important and traditional annual vegetable in many countries, which belongs to the family Cruciferae. The extract of *R. sativus* is known to regulate phase I and II detoxification system inhibiting the proliferation of HepG2 cells, significantly [128]. Its extract contains glucosinolate compounds such as 4-methylthio-3-butenyl isothiocyanate and glucoraphasatin, which imparts anticancer property to *R. sativus* [129]. The extracts of *R. sativus* exhibit effective cytotoxicity against HCT116 colon cancer cell line by inducing apoptosis [130]. Similarly, in the MDA-MB-231 breast cancer cell line, the aerial extract showed effective cytotoxicity via the ErbB-Akt pathway [131].

2.17. Tinospora cordifolia (Willd.) Miers (T. cordifolia). T. cordifolia is an herbaceous vine and belongs to the family of Menispermaceae. It is indigenous to the tropical regions of the Indian subcontinent. It has been used in the Indian Ayurvedic medicines for centuries to treat jaundice, diabetes, and rheumatoid arthritis and is also used as an immunostimulant. *T. cordifolia* is known to have antineoplastic, antioxidant, hepatoprotective, hypolipidemic, and immunologic properties [132]. It is reported to contain a wide array of biologically active compounds which are isolated from the different parts of its body. The main bioactive compounds having therapeutic values include diterpenoid, polysaccharides, lactones, aliphatic compounds, steroids, alkaloids, sesquiterpenoid, and glycosides [133, 134]. The extract of *T. cordifolia* is extensively used in medicinal formulation for its antiperiodic, antispasmodic, antimicrobial, antiosteoporotic, anti-inflammatory, antiarthritic, antiallergic, and antidiabetic properties [135, 136]. *T. cordifolia* is reported to overcome cyclophosphamide (CP) induced toxicities in cancer. In diethylnitrosamine induced hepatocellular carcinoma model in rats, *T. cordifolia* has been reported for its chemo-preventive potential. The chemo-preventive action of *T. cordifolia* is manifested through the decrease in antioxidant activities via superoxide dismutase, catalase, and detoxification enzymes like GPx and GSH, however, increase in the hepatic marker's activities such as lactate dehydrogenase, serum glutamic pyruvate transaminase, serum glutamic oxaloacetic transaminase, and decreased serum transaminase level [136, 137].

2.18. Nigella sativa (N. sativa). This medicinal plant is a member of Ranunculaceae family with annual flowering routine and is native to Eastern Europe and Western Asia. The main bioactive compound which confers anticancer properties to *N. sativa* is Thymoquinone (2-methyl-5-isopropyl-1, 4-benzoquinone). Thymoquinone exhibits cell death of abnormal cells with growth inhibitory activities. According to one study, it is reported that Thymoquinone blocked the tumor growth in murine models [138]. It has been reported that in breast cancer patients, severity of acute radiation dermatitis is reduced after the topical application of *N. sativa* gel [139]. Furthermore, in children with brain tumors, reduction in febrile neutropenia has been reported after taking the *N. sativa* seeds orally [140]. Thymoquinone affects the triggering of numerous upregulation of tyrosine kinases (e.g., PIP3, mTOR, Akt, and MAPK) as well as phosphorylation process of signaling pathways, which are participated in tumor cell proliferation [141, 142]. Thymoquinone also regulates the activation of transcriptional factors (e.g., NF-κB, Nrf2, STAT-3, NF-κB, and Nrf2) counteracting diverse tumorigenic developments, such as cell survival, cell proliferation, cell invasion, inflammation, angiogenesis, and metastasis [142–144]. Thymoquinone shows chemopreventive properties by attenuating the production of proinflammatory mediators (e.g., prostaglandins, chemokines, and cytokines), upregulating the cytoprotective enzymes (e.g., oxidoreductase, superoxide dismutase, and glutathione S-transferase) and downregulating the carcinogen metabolizing enzymes (e.g., CYP 3A4 and CYP 1A2) [142].

Thymoquinone is also reported to sensitize cancer cells to conventional chemotherapy, immunotherapy, and radiotherapy by modulating the resistance mechanisms [145, 146]. Though thymoquinone bears great anticancer properties, its lower efficacy and poor bioavailability are the major constraints as primary therapeutic agent against cancer [147–149].

3. Future Prospective and Conclusion

Medicinal plant-based natural bioactive compounds have played a significant role in human health. As details disputed in this present review, focus was all given in developing new anticancer agents or new treatment strategies protocol against various cancers. As a result, several natural bioactive compounds/metabolites can be investigated/explored in order to explore the mechanistic action and detailed structure types for developing novel anticancer drugs. Though, the drug discovery process, which includes compound isolation, characterization, biological activity determination, preclinical, and clinical trials, is a lengthy and costly process but green alternatives for cancer therapy without any after effects are worth any cost. Current developments in modern techniques and advance instrumentations helped in the identification of several new and more effective novel bioactive drugs obtained from potent medicinal plants, which may further lead in developing effective anticancer compounds. Furthermore, due to their therapeutic potential, they can not only be looked as potential drug against cancer but also promising and effective supplementary foods or nutraceuticals, which are helpful in promoting good health as well as in the management of cancer. Plants are the rich source of bioactive compounds, with active molecules that can act on a variety of biochemical/signaling pathways. Recent researches explore the novelty and effectiveness of anticancer therapeutic drugs derived from plants. Furthermore, some of the medicinal plants and their bioactive compounds have been found to have significant effect against various cancers, primarily breast cancer, lung cancer, leukemia, Kaposi sarcoma, testicular germ cell tumor, Hodgkin's lymphoma, follicular lymphoma, ovarian germ cell tumor, acute lymphoblastic leukemia, acute lymphoblastic leukemia, and others with possible mechanism of action revealed. Hence, it is critical that their identification/discovery be pursued further in order to provide the public with more efficient and compliant therapeutic agents against cancer. The ultimate basis of this review is to reveal the potency of plants as anticancer agents and concluding that most of the research in this field always ends up in early stages without proceeding to identify the full potential of these plants and their therapeutic significance at molecular level, with only a few under *in vivo* experiments and clinical trials as well. Despite of the various potent advantages, there are some concerns regarding the safety and toxicity of medicinal and herbal plants. Some of the medicinal plants as whole can be allergenic or harmful for sensitive populations, while in other cases, certain parts of a plant may be edible and another part may be poisonous. Therefore, toxicological assessment of any medicinal or herbal plant is mandatorily required to identify the adverse effects and to determine limits of exposure level at which such effects occur. Underestimating the toxicological challenges associated with the use of drugs derived from medicinal plants may pose serious health concerns.

Conflicts of Interest

The authors have declared no conflict of interest.

Authors' Contributions

Conceptualization was done by A.J. Siddiqui, F. Bardakci, and M. Adnan; validation was done by S. Jahan, R. Singh, J. Saxena, S.A. Ashraf, A. Khan, R.K. Choudhary, and S. Balakrishnan; formal analysis was done by R. Singh, J. Saxena, S.A. Ashraf, A. Khan, R.K. Choudhary, and R. Badraoui; investigation was done by S.A. Ashraf, F. Bardakci, and S. Balakrishnan; data curation was done by F. Bardakci, S. Jahan, R. Singh, J. Saxena, S.A. Ashraf, A. Khan, R.K. Choudhary, and R. Badraoui; writing—original draft preparation was done by A.J. Siddiqui, S. Jahan, and M. Adnan; writing—review and editing was done by A.J. Siddiqui, F. Bardakci, M. Adnan, S. Jahan, R. Singh, J. Saxena, R. Badraoui, and S. Balakrishnan; visualization was done by A. Khan, R.K. Choudhary, R. Badraoui, and S. Balakrishnan; supervision was done by A.J. Siddiqui and M. Adnan; project administration was done by A.J. Siddiqui. All authors have read and agreed to the published version of the manuscript.

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Research Article

Cytotoxic and Antioxidant Effects of Phoenix dactylifera L. (Ajwa Date Extract) on Oral Squamous Cell Carcinoma Cell Line

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Aim. The aim of the current study is to investigate the antioxidant and apoptotic potential of Ajwa date flesh (ADF) and Ajwa date pit (ADP) extract on human squamous cell carcinoma cell line (HSC-2). *Method*. ADF and ADP were extracted with a solvent extraction method using hexane, acetone, and ethanol, which were then subjected to antioxidant assay by 2,2-diphenyl-1-picrylhydrazyl (DPPH). HSC-2 cells were then treated with different concentrations of ADF and ADP extract for 24, 48, and 72 hours. MTT assay was performed to assess the antiproliferative effect, and Annexin V-FITC was used for the detection of cellular apoptosis. *Results*. Acetone extracts of ADF and ADP had the highest radical scavenging and antioxidant activities followed by the ethanolic extracts, whereas ADP appeared to have significantly higher antioxidant effects than ADF. MTT assay demonstrated that acetone extracts of ADF and ADP were significantly cytotoxic against HSC-2 cells in a dose- and time-dependent manner. The half inhibitory concentration (IC50) of ADF was found to be 8.69 mg/ml at 24 h, and the maximum cell growth inhibition was observed at 50 mg/ml. The IC50 for the ADP was found to be 0.97 mg/ml at 24 h, and the maximum cell growth inhibition was observed at 5 mg/ml. Statistical analysis of the flow cytometry assay showed that the treatment with ADF and ADP extracts had a significant apoptotic effect which occurred in a dose-dependent manner. HSC-2 cells were seen in the late apoptotic stage with higher doses of ADF and ADP extract. ADP extract demonstrated higher apoptotic activity than ADF extract. In addition, combined treatment of ADF and ADP was also performed on HSC-2 cells which demonstrated higher apoptotic activity when compared to the single extract. *Conclusion*. Ajwa date fruit has a promising cytotoxic effect by inhibiting the growth and proliferation of OSCC cells and inducing cell death by apoptosis.

1. Introduction

Oral squamous cell carcinoma (OSCC) is the 6th most common cancer around the world. It has been documented that 90% of all oral malignancies are squamous cell carcinoma (SCC) [1]. With an increase in the prevalence and mortality rate, there were an estimated 377,713 new cases and 177,757 deaths from oral cancer in 2020 [2]. The incidence of the disease is influenced by several factors such as use of tobacco, betel quid chewing, alcohol drinking, infection with HPV, genetics, radiation, unhealthy diet, and physical inactivity [3]. The 5-year survival rate is 50%, but if detected in

early stages, it is increased to 60-80% [4]. Despite advancement in surgical approach and radiation therapy, the adverse effects of these treatments include anaemia, loss of appetite, and peripheral neuropathy leading to poor quality of life. Therefore, there is a need to develop an effective treatment with potent anticancer activity and less adverse effects [5].

A new approach in the management of oral cancer treatment is by antioxidant therapy. Reduction in oxidative stress which damages the intracellular structure and DNA of cell can be achieved by antioxidants, which are responsible for the removal of free radicals. Studies have found that date fruit scavenges the free radicals and prevents the occurrence of macromolecular changes in living systems [6]. Studies have also found that Ajwa date flesh and pit have shown gastroprotective [7], hepatoprotective [8], antidiabetic [9], and anti-inflammatory properties [10] due to their strong antioxidant, antimutagenic, and anticancerous activity which can be attributed due to the presence of phenolic compounds and flavonoid glycosides [11] [12]. Ajwa date flesh and pit are also attributed for antimicrobial [13], antifungal [14], and antiviral activities [15]. In the religion of Islam, Ajwa dates have remarkable status as narrated in Hadith by Sahih Al-Bukhari: The Prophet (peace be upon him) said "Whoever eats seven Ajwa dates every morning will not be harmed by poison or witchcraft all that day until night comes" [16].

Ajwa are soft fleshy dates with blackish-brown colour. The edible part is high in sugar content and moisture whereas the pit part is high in crude protein [16], while both parts contain abundant number of dietary fibres and high amount of essential minerals which are necessary for the skeletal growth and maintenance of cellular functions in human body in contrast with other date varieties [12, 16–18]. Provitamin A, C, D, E, and K; riboflavin (B2); pyridoxine (B6); and niacin (B3) were also found in Ajwa date flesh [19–21]. Date fruits also contain sulphated-flavanol glycosides. This form of flavanols was not detected before in any fruit as well as vegetables [22, 23].

Ajwa date fruit reported to have high amount of carotenoids like beta-carotene and lutein and polyphenols like quercetin, isoquercetin, luteolin, apigenin, rutin, and anthocyanins when compared to other varieties [24]. These phytochemicals are known for their antioxidant property which help in the prevention and treatment of cancer through inhibition of cell cycle progression and induction of apoptosis by modulating signalling pathways which can regulate intracellular reactive oxygen species (ROS), Bcl-2/Bax, and p53/p21 pathways [25, 26].

The anticancerous mechanism was investigated in a study on human breast adenocarcinoma (MCF7) cell line with methanolic extract of Ajwa date. The result showed significant inhibition of cell proliferation in a dose- and time-dependent manner. Flow cytometric analysis showed that the cell death was due to apoptosis [27]. In another study, date seed oil also has shown chemoprotective effects and can be used in the prevention of skin cancer caused by UV radiation [28, 29]. Few other *in vitro* studies showed similar results of cytotoxic properties of Ajwa date extract with aqueous acetone on cervical (HeLa) cell line [30], Ajwa date extract with ethanol on hepatocellular carcinoma (HepG2) cell line [31], and lung cancer (NCI-H460) cell line [32].

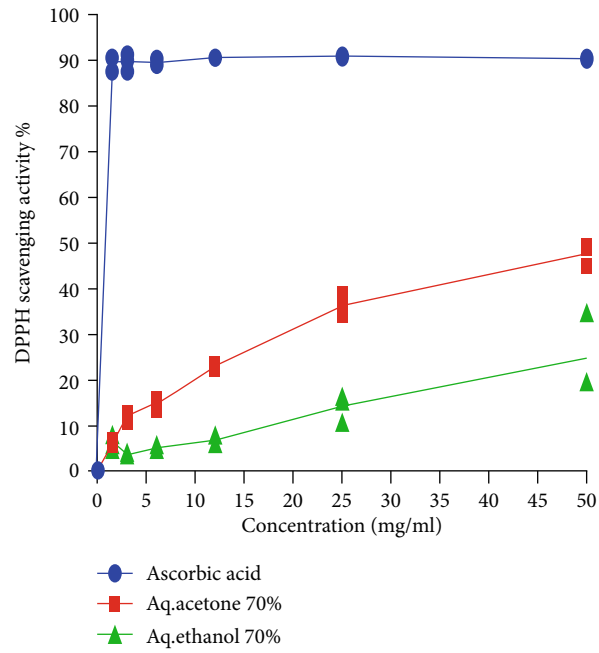


Figure 1: DPPH scavenging activity of different solvents of ADF extract at different concentrations. The curve for ascorbic acid has been used as a standard. Each value represents the mean of three independent experiments ($n = 3$).

The extraction of different phytochemical compounds is influenced by the polarity of the solvent. Anthocyanins are best extracted by ethanol [33], whereas acetone was the best solvent for the extraction of phenols and flavonoids [34]. Since polyphenols, flavonoids, and flavones attributed to the Ajwa date antioxidants and tissue protective properties, it is important to select a single solvent which can yield the maximum antioxidant from the fruit [35]. In the previous literature, different solvents have been used to extract different bioactive components according to their research needs; therefore, three solvents were selected in the present study to extract the maximum amount of polyphenols and flavonoids. To this date and to our best knowledge, no study has been conducted to oversee the cytotoxic and apoptotic effects of Ajwa date flesh and pit on human oral squamous cell carcinoma cell line (HSC-2).

2. Materials and Methods

2.1. Preparation of Ajwa Date. Ajwa dates are only cultivated in the outskirts of city of Madinah Al Munawwarah. Four kg of Ajwa dates was purchased from the "Tamar Market" of Al-Madinah Al Munawwarah, Kingdom of Saudi Arabia. Medium-sized Ajwa dates without any visible physical damage were selected and washed, first with tap water and then with distilled water three times under sterile environment to remove all the dust and soil particles; then dried with sterile cotton cloth; and air-dried under shade for one night. Ajwa dates were then manually pitted, and the seeds were washed again to remove any remaining date flesh and air-

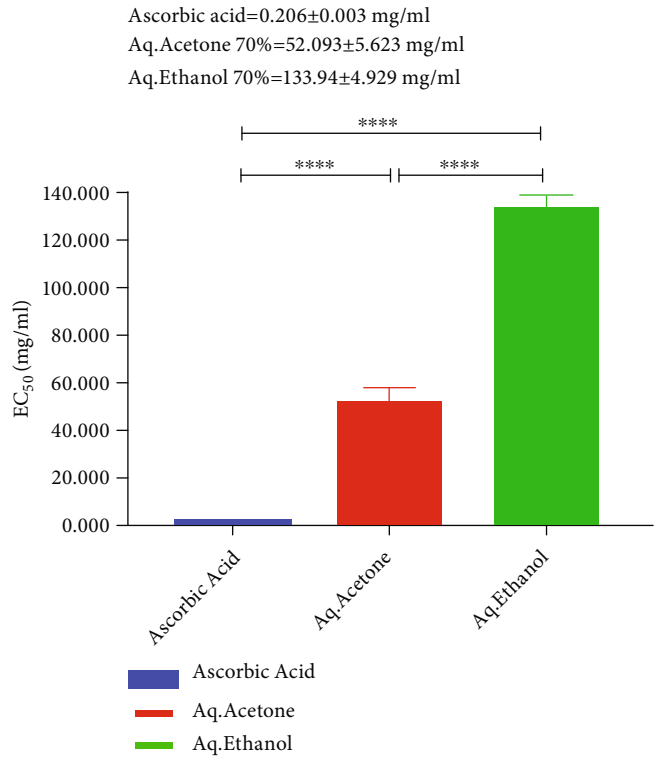


FIGURE 2: Comparison of EC_{50} value of aq. acetone and aq. ethanol extract of ADF with the standard EC50 value of ascorbic acid. ****Significant difference from control at $p < 0.05$.

dried for another night. Ajwa date flesh was chopped to small pieces approximately 1 cm each using a surgical blade no. 10. Ajwa pits and the cut date flesh were then further dried completely using a freeze-drying method for a week separately in order to stabilize the samples and prevent microbial spoilage and hydrolytic rancidity [36]. Date pits were milled using a laboratory milling grinder with 30~300-micron finesse. The flesh part was crushed coarsely using a pestle and mortar. The powdered pits and flesh were weighed and made into aliquots of 50 g and sealed into air-tight plastic bags stored at -40°C in dark until required for extraction.

2.2. Extraction of Date Flesh and Seed. In this study, different solvents such as n-hexane, acetone/H_2O (70 : 30, *v*/*v*), and ethanol (70 : 30, *v*/*v*) were used successively in order to isolate a wide range of antioxidant compounds present in Ajwa date's flesh and pits. In this study, the order in which extraction was performed was n-hexane, acetone 70%, and ethanol 70%. Ajwa date's flesh and pit extractions were performed separately following the protocol described previously by [27, 37] with slight modifications.

A portion of freeze-dried contents (15 g) was extracted in n-hexane (150 ml) with a ratio of 1 : 10 (weight to volume) for 48 hours at room temperature in a flat bottom flask on a shaking incubator. Following extraction, the resultant extract was filtered using Whatman No. 1 filter paper. The remaining residue was further extracted in acetone/H_2O (70 : 30) for 48 hours at room temperature with continuous agitation. The solution was filtered and evaporated under vacuum using a concentrator to give a dark brownish extract. Remaining insoluble residue was subjected to ethanol/H_2O (70 : 30) extraction for 48 h at room temperature. The solution was filtered, and the filtrate was evaporated under vacuum to give dark brown concentrate. All the crude extracts were frozen, and the H_2O removed by freeze-drying to yield a brown solid. All the extracts of Ajwa date's flesh and pits were weighed, sealed, labelled, and stored at -20°C in 50 ml tubes for analytical purposes.

2.3. Antioxidant Properties of Date Extract

2.3.1. DPPH Assay. To evaluate the antioxidant capacity of Ajwa date's flesh and seed extracts from three different solvents, diphenyl picrylhydrazyl (DPPH) assay was carried out to obtain the highest biologically active extract according to the previously described protocol [38]. The working solution of DPPH in methanol was prepared daily for the measurement of antioxidants in the extracts using a UV spectrophotometer. To prepare the 0.1 mM of DPPH (molecular weight 394.32 g/mol) solution, 3.94 mg of DPPH was dissolved in 100 ml of methanol in a flask which was covered by aluminium foil. Three ml of this solution was then mixed with 100 μl of various concentrations of sample extract solution in disposable microcuvettes. The samples were kept in a dark place for 30 min at room temperature before being measured for absorption at 517 nm using the spectrophotometer. A blank sample containing 3 ml of DPPH solution was measured daily to obtain an absorbance of 0.0 ± 0.02 units at 517 nm. Ascorbic acid was used as a

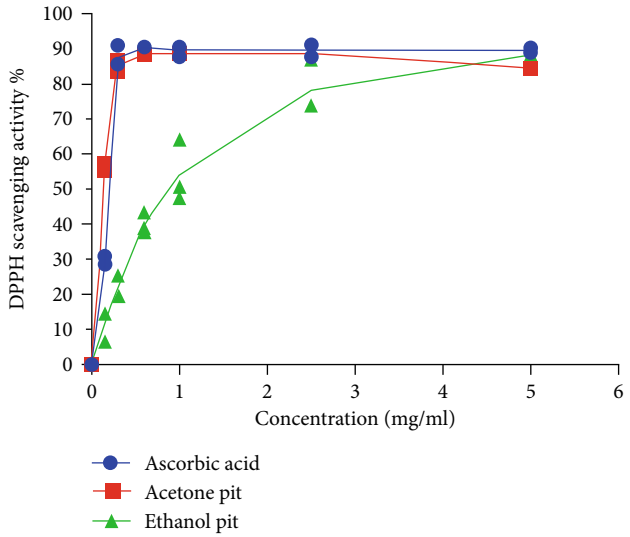


Figure 3: DPPH scavenging activity of various fractions of Ajwa date pits at different concentrations. The curve for ascorbic acid is used as a standard. Each value represents the mean of three independent experiments ($n = 3$).

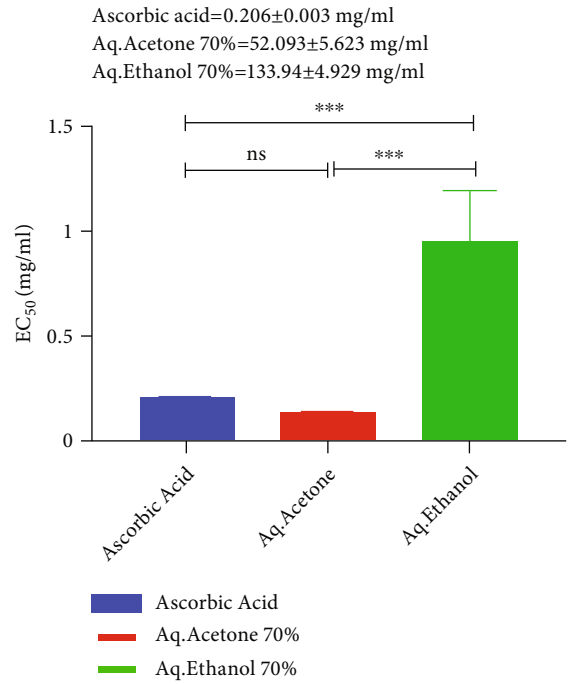


Figure 4: Comparison of EC50 value of aq. acetone and aq. ethanol extract of ADP with the standard EC50 value of ascorbic acid. ***Significant difference from control at $p < 0.05$; NS: no significant difference from control.

standard reference for the comparison of results. A standard curve was prepared for calibration using six concentrations of ascorbic acid ranging from 1.5 to 50 mg/ml. The total antioxidants are expressed as milligrams per milliliter of ascorbic acid. The experiment was run in triplicate, and average was taken to calculate the radical scavenging activity using the following formula:

$$\%\text{inhibition} = \left[\frac{(A_b - A_s)}{A_b}\right] \times 100, \quad (1)$$

where A_b is absorbance of control and A_s is absorbance of the sample.

2.4. Cytotoxic Properties of the Date Extract

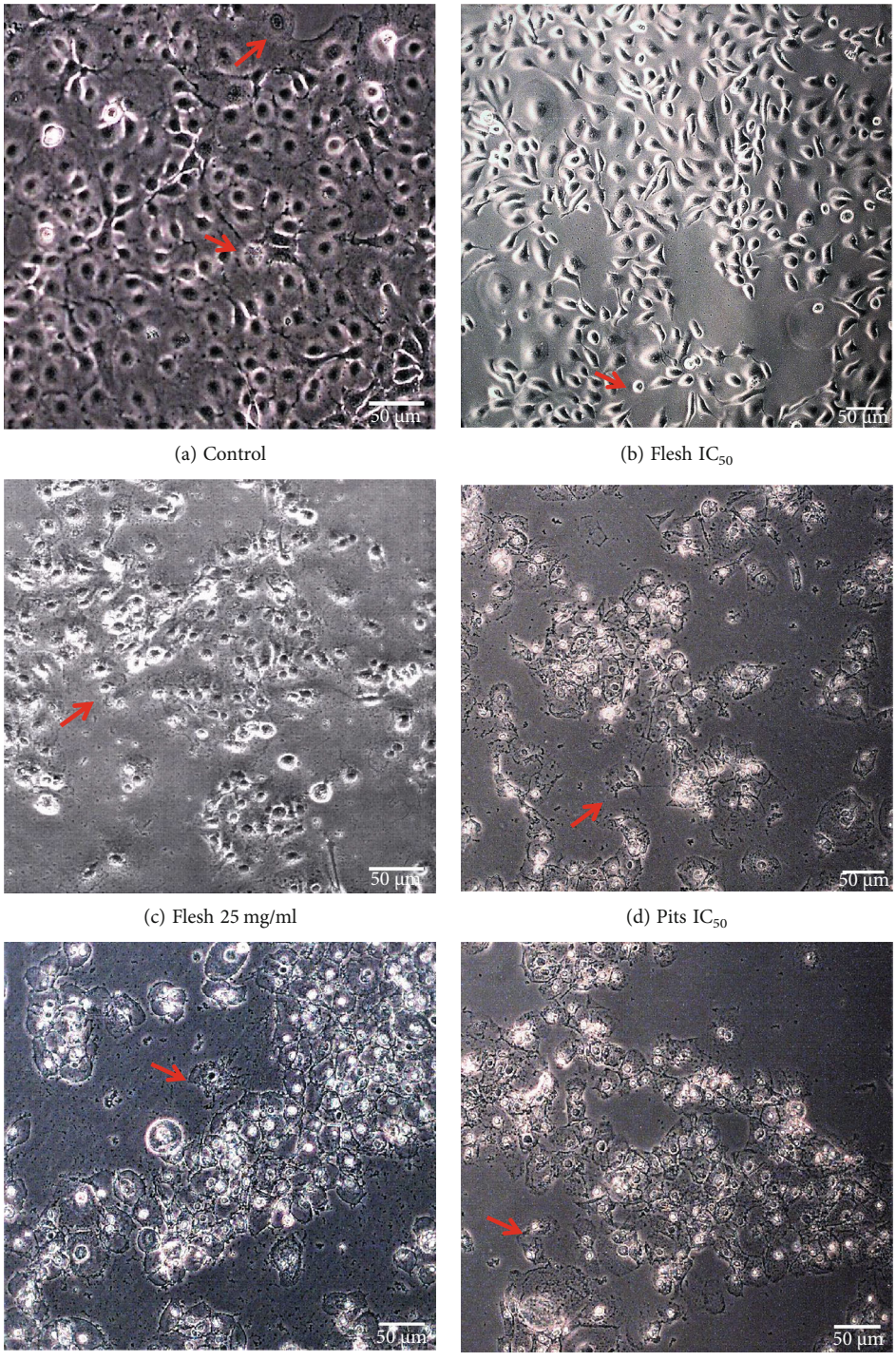
2.4.1. HSC-2 Cell Culture. HSC-2, RCB-1945, is a human oral squamous carcinoma cell line, which was purchased from Riken Cell Bank, Japan. HSC-2 cells were cultured in minimum essential medium (MEM) containing 2 mM L-glutamine, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin. The cells were incubated in 5% CO_2 at 37°C incubation in a humidified CO_2 incubator. Cells were maintained as a monolayer, and cell passage was performed every 3rd or 4th day.

2.5. Evaluation of Morphological Changes of HSC-2 Cells. Cells were cultured in 6-wells (10 mm) cell culture petri dishes at a seeding density of 2×10^5 cells/well. After 24-hour attachment, the cells were treated with different concentrations of Ajwa date's flesh and pit extracts separately and in combination, whereas untreated cells served as control. The morphological changes were observed under an inverted microscope, and images were taken for the comparison with the untreated cell images at 48-hour treatment.

2.6. Assessing Cell Viability by MTT Assay. In this study, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the cytotoxic effect of Ajwa date's flesh and pit extracts on oral squamous cell carcinoma. A range of concentrations (0.8, 1.5, 3.1, 6.3, 12.5, 25, and 50 mg/ml) of ADF extract [27, 31, 39] and (0.08, 0.31, 0.63, 1.15, 1.25, 2.5, and 5 mg/ml) of ADP extract [40, 41] were selected with 70% aq. acetone.

The HSC-2 cells were harvested and seeded in a 96-well plate at a cell density of 5×10^3 cells/well. Cell's attachment was confirmed under a microscope after 24 hours; the cells were then treated with different concentrations of ADF and ADP extracts separately. Control wells were treated with the same amount of complete growth media only. For every treatment and untreated control group, complete growth media without cells were added as a blank to reduce the background absorbance values. For every concentration of ADF and ADP extracts, 6 wells were used, three with treatment and three as blanks. All the experiments were carried out three times independently with 3 replicates in each experiment.

The cells were grown with sample extracts up to three different time points (24 h, 48 h, and 72 h) after which MTT assay was performed by removing the medium gently and adding 10 μl of MTT solution with a final concentration of 5 mg/ml per well, and incubated at 37°C for about 4 hours until the purple crystals were formed. After that, the MTT solution was discarded from every well and 100 μl of DMSO was added to dissolve the crystals. The 96-well plate was mounted on a microplate shaker and shaken for 15 minutes until the crystals completely dissolved. The absorbance value



(a) Control

(b) Flesh IC_{50}

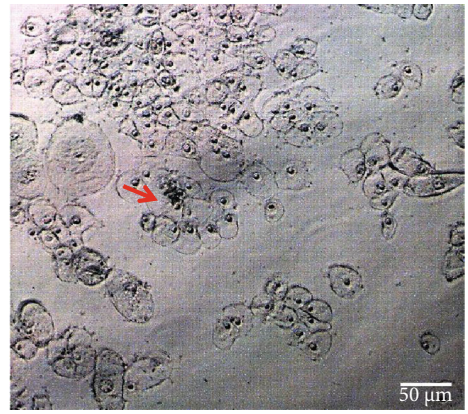
(c) Flesh 25 mg/ml

(d) Pits IC_{50}

(e) Pits 2.5 mg/ml

(f) Flesh IC_{50} + pits IC_{50}

Figure 5: Continued.



(g) Flesh 25 mg/ml + pits 2.5 mg/ml

Figure 5: Morphological appearance under an inverted light microscope after 48 hours. Mitotic figures can be seen in (a), cell shrinkage and cell detachment can be seen in (b) and (c), and membrane disruptions, cytoplasmic condensation, and cell death can be seen in (d), (e), (f), and (g); magnification 100x.

for each well was determined at an optical density at 570 nm wavelength using an ELISA microplate reader [42]. The 50% inhibitory concentration (IC_{50}) of the ADF and ADP extract was also calculated at 24 h.

The cell viability (CV) percentage after treatment with ADF and ADP extract was calculated with the formula below:

$$\mathrm{CV}(\%) = \frac{\text{absorbance of treatment cells-absorbance of blanks}}{\text{absorbance of control cell-absorbance of blanks}} \times 100. \quad (2)$$

2.7. Detection of Apoptosis by Flow Cytometry Assay. Annexin V-FITC (fluorescein isothiocyanate) Apoptosis Detection Kit I was used to quantify apoptotic cells by flow cytometry following the manufacturer's instructions. For negative control, untreated cells were used. Briefly, HSC-2 cells at 2×10^5 cells/ml density were incubated for 24 h to allow adherence of cells to the 6-well culture plate. After 24 hours, the cells were incubated with 8.69 mg/ml (IC_{50}) and 25 mg/ml of ADF extract and 0.97 mg/ml (IC_{50}) and 2.5 mg/ml of ADP extracts for 24, 48, and 72 hours. After the given time point, the cells were harvested by washing with PBS and trypsinizing with 400-450 μl of trypsin. Cells were then centrifuged with 1 ml of cold PBS twice and once with 100 μl of 1X binding buffer solution for 5 minutes each time at 157 × g. The cells were stained with 5 μl Annexin V-FITC and 5 μl Propidium Iodide (PI) for 15 min at 25°C in the dark and resuspended in 400 μl of 1X binding buffer solution and immediately analysed by BD Accuri ™ C6 flow cytometry in 5 ml round-bottom polystyrene FACS tubes. Wavelengths of 533 nm and 585 nm were used for Annexin V-FITC and PI, respectively. 10,000 events per sample were recorded on forward scatter versus side scatter plot using BD Accuri™ C6 software. Control samples were prepared separately for the purpose of recording the HSC-2 cells according to their granularity and size. Cells with PI-only stain represent necrosis, PI and Annexin-V represent late apoptosis, cells stained with Annexin-V only represent early apoptosis, and unstained cells were evaluated as viable healthy cells.

2.8. Combination Treatment. A combination treatment of both ADF and ADP extracts was performed together to analyse if there was any synergistic effect in apoptosis of the cells.

IC_{50} concentrations from the MTT assay of ADF and ADP were combined together (8.69 mg/ml + 0.97 mg/ml) as a single treatment, and the results were recorded.

Likewise, next higher concentrations were combined for both the extracts (25 mg/ml + 2.5 mg/ml) and used as a single treatment to observe if it had significantly higher apoptotic effect than if used separately.

2.9. Statistical Analysis. The statistical analysis was carried out using SPSS 24 version. To compare the difference between the control and the treated groups for DPPH, cell proliferation and apoptosis assay. One-way ANOVA was conducted followed by either Tukey's post hoc or Dunnett's multiple comparison test. The IC_{50} values for DPPH and MTT assay were calculated using nonlinear regression analysis function with Prism GraphPad software (version 7). All experimental data was represented as the mean ± standard deviation (SD) of three independent experiments which were performed in triplicate. A p value of less than 0.05 ($p < 0.05$) was considered statistically significant.

3. Results

3.1. DPPH Assay. Assessment of the total antioxidant contents of the Ajwa date flesh's extract was performed. The assay could not be performed with n-hexane solvent due to the insufficient amount of extracted residue present to make a working solution to perform the assay. Therefore, antioxidant activity from the acetone/H_2O (70 : 30, v/v) and ethanol/H_2O (70 : 30, v/v) was studied.

The results of ADF sample of aq. acetone 70% and aq. ethanol 70% of 1 to 50 mg/ml concentrations are shown in Figure 1. Data shows that the percentage of inhibition of

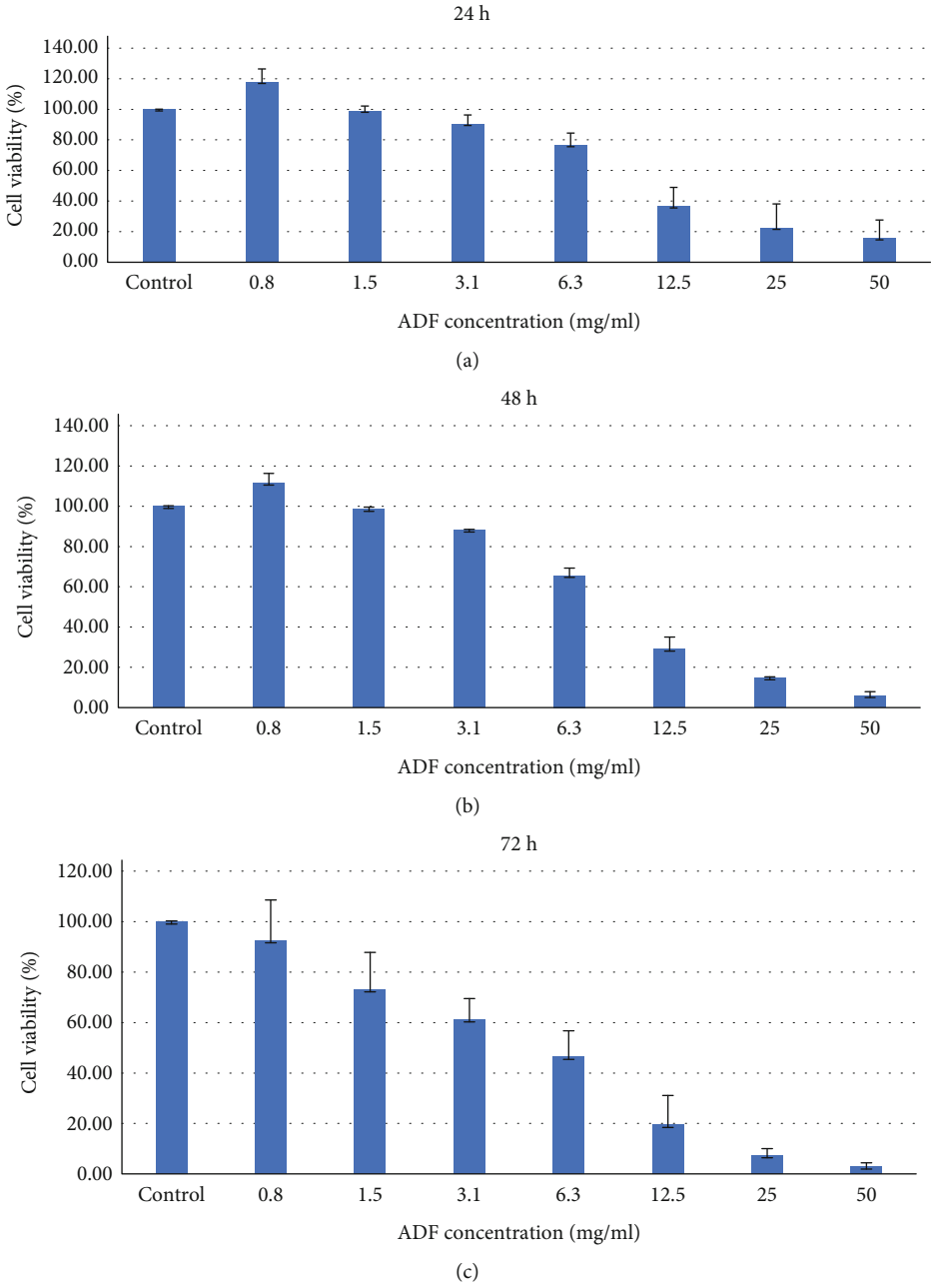


Figure 6: Inhibition of proliferation of HSC-2 cell. MTT assay of HSC-2 cells treated with ADF extract at different concentrations at (a) 24 h, (b) 48 h, and (c) 72 h. These decreases in percentage of cell viability were statistically significant. The values are expressed as mean SD from triplicate samples of three independent experiments. $*$ indicates $p < 0.05$.

aq. acetone 70% ADF extract was higher than that of the aq. ethanol 70% ADF extract. The curve for ascorbic acid also showed the same inhibition pattern, but at a very low concentration of 1.5 mg/ml, it reached the highest inhibition percentage of 89.8%.

The graph (Figure 1) presented showed that the extract concentrations of both solvents are proportional to the percentage of inhibition, which means the greater the concentration of extracts, the greater the DPPH scavenging activity. The inhibition percentage was significantly greater for the aq. acetone extract of ADF (median = 15.1%) than the aq. ethanol extract of ADF (median = 5.6%) ($p = 0.011$).

3.2. Determination of EC_{50} Value for Ajwa Date Flesh Extract with Various Solvents. EC_{50} is the dose concentration of the sample required to reduce 50% of the free radicals of DPPH. The half-maximal effective concentration (EC_{50}) of 70% aq. acetone extract of ADF was 52.09 mg/ml, and EC_{50} of 70% aq. ethanol extract of ADF was 133.94 mg/ml. Ascorbic acid $EC_{50} = 0.206$ mg/ml. The graphical representation is shown in Figure 2. These results suggest that 70% acetone extract has more antioxidant activity than the 70% ethanol extract of flesh sample. The high EC_{50} value of 70% ethanol extract suggests the minimal amount of antioxidant present in this extract.

3.3. Assessment of the Total Antioxidant Contents of the ADP. For ADP sample, concentrations ranging from 0 to 5 mg/ml were selected for both solvents. 70% aqueous acetone extract has greater DPPH free radical scavenging activity at a lower concentration than 70% aqueous ethanol extract of ADP, but the highest average yield of the total antioxidants for both the solvent samples was reached at the concentration of 5 mg/ml as shown in Figure 3. At the $p < 0.05$ level of significance, the results showed that there is significant difference in the inhibition percentage ($p = 0.04$) between the 70% acetone (median = 86.2%) and 70% ethanol (median = 39.1%) solvent extract.

3.4. Determination of EC_{50} Value of Ajwa Date Pits with Various Solvents. The EC_{50} of 70% aq. acetone extract of ADP was 0.153 mg/ml, and that of 70% aq. ethanol extract of ADP was 0.954 mg/ml. The EC_{50} value of ascorbic acid was 0.206 mg/ml, as shown in Figure 4.

3.5. ADF and ADP Induced Changes in Cell Morphology. Images taken from the light microscope of HSC-2 cells of control sample demonstrated characteristic of epithelial nature and growth proliferation as a monolayer. The cells appeared to be attached together in an ovoid shape with a large central nucleus; dividing cell can also be seen with two or more nucleoli in Figure 5(a). In contrast, the ADF and ADP extract-treated cells showed mild to severe decrease in cell numbers (Figures 5(b)–5(g)) which was dose dependent leaving behind very few live cells. At a concentration of ADF extract IC_{50}, cell shrinkage and partial cell-to-cell detachment can be seen; cells have also started losing their shape. At a concentration of ADF extract 25 mg/ml, more drastic changes can be seen; cells have become rounded in shape with complete cell-to-cell detachment, with decrease in number of viable cells. For ADP extract at a concentration of $IC_{50,}$ cytoplasmic vacuolization, nuclear condensation, and cluster shrinkage can be seen. At a concentration of 2.5 mg/ml, a lot of cellular fragmentation can be seen with very few viable cells. For the combination treatment with ADF and ADP extract, drastic morphological changes can be seen in Figures 5(f) and 5(g)); nuclear condensation, cell membrane blebbing, and fragmentation are vastly present with very few viable cells.

3.6. ADF Inhibited HCS-2 Cell Proliferation. The MTT assay results from the present study demonstrated HSC-2 cell growth inhibition following the treatment with ADF extract. At 24 h treatment period, ADF extract reduced cell viability to 99, 90.3, 76.5, 36.5, 22.4, and 15.6% at 1.5, 3.1, 6.3, 12.5, 25, and 50 mg/ml, respectively. Negative correlation can be seen between the concentration of ADF extract and viable cells of OSCC. As the concentration of the ADF extract increases from 1.5 to 50 mg/ml, the percentage of viable cells decreased from 99% to 15.6% indicating a dose-dependent manner. At 48 h treatment period, ADF extract exerted a more pronounced effect, drastically reducing the viability of treated cells to 98.5, 88.6, 65.6, 29, 14.8, and 5.9% at 1.5, 3.1, 6.3, 12.5, 25, and 50 mg/ml of extract, respectively. Moreover, a further decrease in cell viability percentage was seen at 72 h with a percentage of 66.5, 61.1 46.3, 19.4, 7.3, and 2.9% at 1.5, 3.1, 6.3, 12.5, 25, and 50 mg/ml, respectively, stipulating that ADF extract is more toxic at higher concentrations, as presented in Figure 6. From the above results at 50 mg/ml concentration, the cell viability decreased to 15.6% at 24 h, 5.9% at 48 h, and 2.9% at 72 h. Therefore, the cell viability data proposed that treatment with ADF extract significantly reduced HSC-2 cell growth in both dose- and time-dependent manners, signifying its ability to impair proliferation potential. IC_{50} value is estimated to comprehend the basic pharmacological and biological characteristics; the lower the IC_{50} value, the more potent the drug is [43]. The IC_{50} value was determined to be 8.69 mg/ml at 24 h duration, in Figure 7.

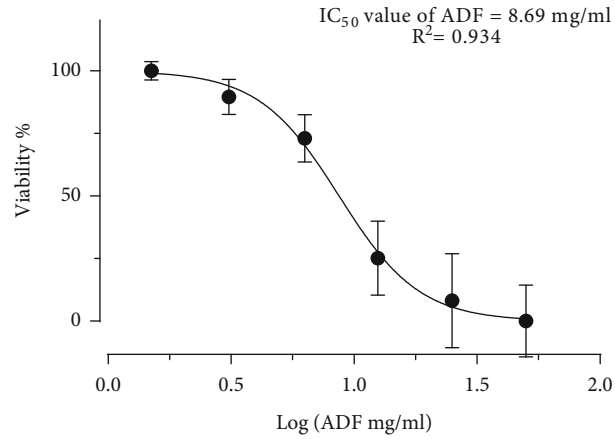


Figure 7: Determination of IC_{50} value of (ADF) on HSC-2 cells.

3.7. ADP Inhibited HSC-2 Cell Proliferation. MTT assay demonstrated HSC-2 cell growth inhibition following the treatment with ADP extract in a dose-dependent manner (Figure 8). Ajwa date pit extract showed similar but enhanced results compared to the flesh part with significant cytotoxic effect on the viability on HSC-2 cells. At 24 h treatment incubation time, ADP extract reduced cell viability to 82.2, 73.7, 54.9, 40.7, 22.3, 15.1, and 6.6% at 0.08, 0.3, 10.6, 31.1, 51.2, 2.5, and 5.00 mg/ml concentration of ADP extract, respectively. At 48 h treatment period, ADP extract reduced cell viability 79.3, 70.1, 46.5, 25.3, 14.2, 10.6, and 3.7% at 0.08, 0.3, 10.6, 31.1, 51.2, 2.5, and 5.00 mg/ml concentration of ADP extract, respectively, as shown in Figure 8. Thus, the data demonstrated cell growth inhibition in a dose- and time-dependent manner. The significant decrease in percentage of cell viability started from 0.63 mg/ml concentration at 24 h and at 48 hours and 1.25 mg/ml at 72 h. The IC_{50} value was determined to be 0.97 mg/ml at 24 h duration from Figure 9.

3.8. ADF and APD Induced Apoptosis in HSC-2 Cells. The percentage of early apoptotic and late apoptotic cells increased in the treated group when compared to that of the early and late apoptotic cells of control cells. Among the various extracts, ADP 2.5 mg/ml showed the highest percentage of late apoptotic cells and combination of ADF and ADP IC_{50} concentration showed the highest percentage of early apoptotic cells at 24 h. At 48 h, ADP IC_{50} showed the

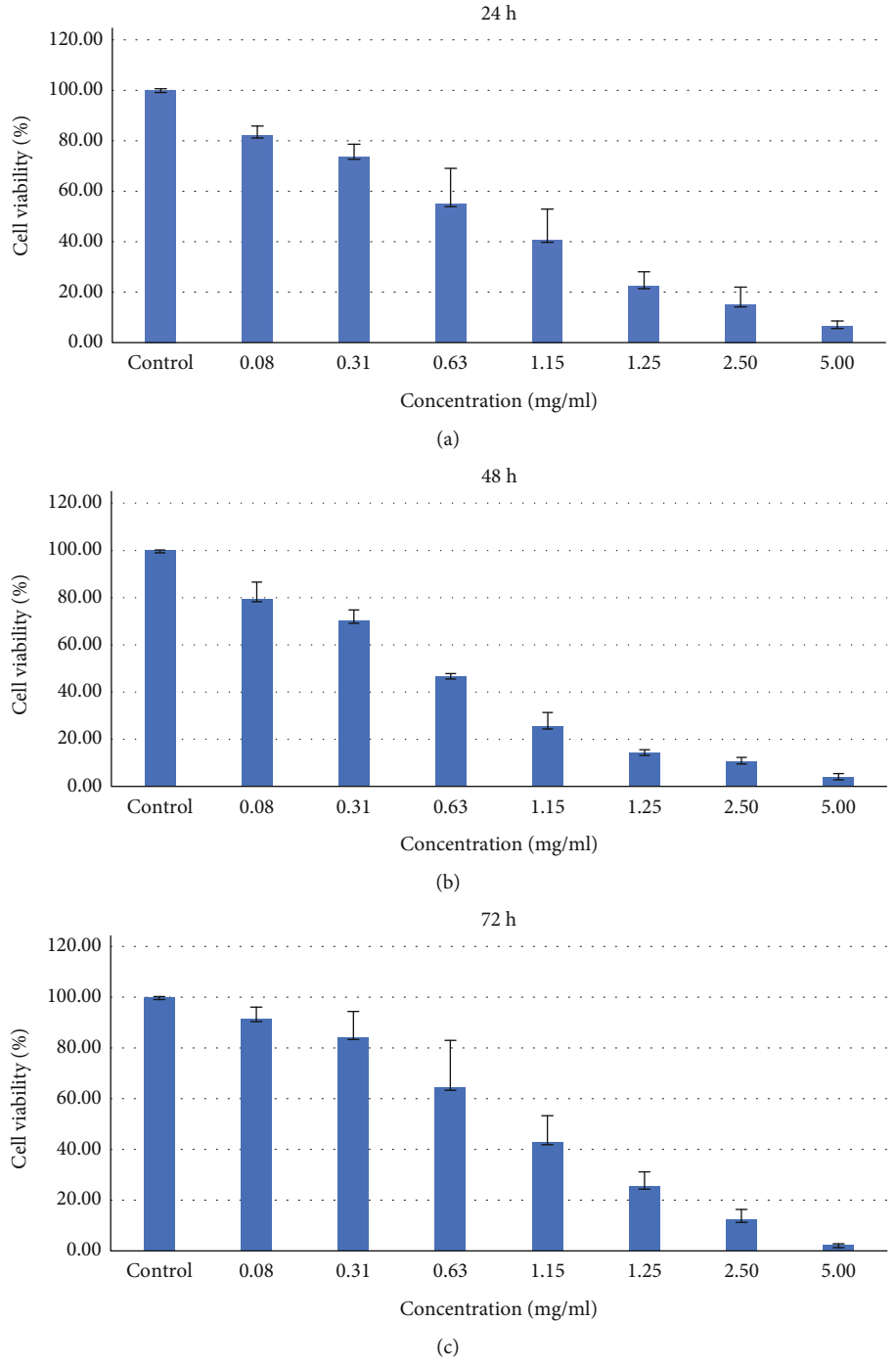


Figure 8: MTT assay of HSC-2 cells treated with ADP extract at different concentrations at (a) 24 h, (b) 48 h, and (c) 72 h. The values are expressed as mean SD from triplicate samples of three independent experiments. $*$ indicates $p < 0.05$.

highest percentage of late apoptotic cells and combination of ADF 25 mg/ml+ADP 2.5 mg/ml showed the highest early apoptotic cells. At 72 h, the increase in total apoptotic cells was $40 \pm 47.09\%$ and $62.37 \pm 50.09\%$ for IC_{50} (8.69) mg/ml and 25 mg/ml of ADF, respectively; for ADP, total apoptotic cells were $82.13 \pm 50.7\%$ and $69.77 \pm 37.03\%$ for IC50 (0.97) mg/ml and 2.5 mg/ml, respectively, compared to control $10.83 \pm 3.87\%$. (Figures 10 and 11).

When the combination of both ADF and ADP extracts as a single-treatment ADF and ADP IC_{50} (8.69 + 0.97) mg/ml and ADF and ADP (25 + 2.5) mg/ml is given, the result of total apoptotic cells was76.57 ± 50.21 % and 75.47 ± 21.64 % (Figures 10 and 11(a)–11(d)).

4. Discussion

The DPPH results of ADF and ADP extracts demonstrated that when acetone was used as a solvent, higher antioxidant activity was obtained in both flesh and pits compared to the values obtained with ethanol at the same solvent

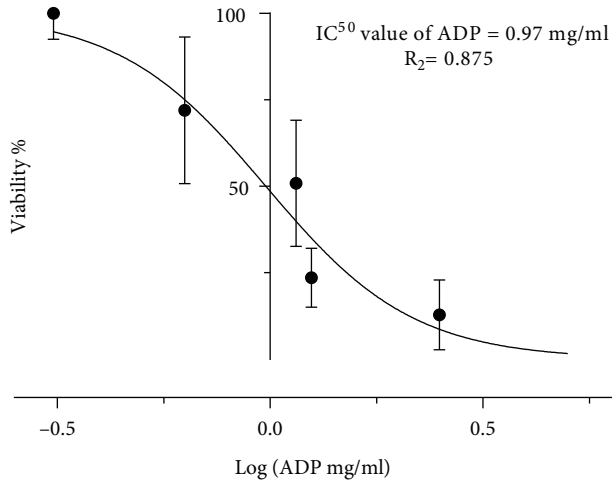


FIGURE 9: The graph was constructed to determine the IC_{50} value of ADP extract on HSC-2 cells.

concentrations (70%). These results are in line with a previous study conducted by Nematallah et al. in 2018 where 50% aq. acetone yielded the highest antioxidant activity in Ajwa date, followed by ethanolic extract. The variations in the values can be due to the difference in extraction procedure [44]. Another important finding observed from the DPPH assay was that the pit extract exhibited higher antioxidant activity with both the solvents when compared with the flesh part. These results are in support with a study reported by Maqsood *et al.* where the concentration of acetone and ethanol between 60 and 80% yielded the highest DPPH free radical scavenging activity from a date pit [45]. Therefore, it can be concluded that pit appeared to be a richer source for phenols and flavonoids; these results can be supported by previous studies, where date pits are the highest source of total polyphenols among tea, flaxseed, nut seeds, grapes, and even date flesh [46, 47]. Date fruit flesh also contains polyphenols like quercetin and kaempferol [48], which possess anticancer activity against oral squamous cell carcinoma [49, 50]. Therefore, it can be said that the anticancer effect of Ajwa date extract against HSC-2 cells might be due to the integrated or collective effect of the potential bioactive components of Ajwa dates. The anticancer activity of these bioactive compounds can be mediated by several molecular mechanisms including free radical scavenging activity, deactivation of carcinogenic metabolites, antiproliferation, induction of apoptosis, and cell cycle arrest [51].

One of the hallmarks of cancers is its ability to replicate and invade through escaping apoptosis, being insensitive to antigrowth signals, and self-sufficient in growth signals to proliferate uncontrollably. Hence, finding new drugs and treatments, targeting various pathways in the induction of apoptosis and inhibition in the proliferation, plays an important role in the treatment of cancers. Interestingly, the morphological data of the present study revealed ADF- and APD-mediated changes which are indicative of apoptosis. Treated cells appeared shrunken, nonadherent, partially detached, and rounded in shape, with also a decrease in cell number. As the concentration of ADF and ADP extracts increased, more drastic changes were observed like cluster shrinkage, membrane blebbing, and cellular fragmentations leading to cell death, exhibiting a dose-dependent severity. These are typically initial characteristic features of apoptotic cell death [52]. In addition, both MTT and annexin V-FITC costaining with PI assays demonstrated inhibition in cell growth following treatment with ADF and ADP extract, supporting the morphological observations in the HSC-2 cells. In previous studies, apoptosis or initiation of apoptotic pathways has been induced by bioactive components and secondary metabolites of natural products [53].

Interestingly, in the present study, the data from MTT assay showed that, at a concentration of 0.8 mg/ml of ADF extract, there was an increase in cell viability percentage of 117.9 and 111.4% at 24 and 48 h of incubation period, respectively, when compared to the untreated control group. This could be explained by a phenomenon known as hormesis, where some cells might try to adjust to the toxic environment at very low dose resulting in a higher MTT signal compared to the control [54]. ADP extract showed increased cytotoxic effect on the viability on HSC-2 cells, which can mainly be attributed to the presence of high amount of phenolics, flavonoids, and vitamin C in the seeds compared to the flesh part [46]. The date seeds contain high amount of total polyphenols, close to 3942 mg/100 g, whereas date flesh contains 239.5 mg/100 g wet weight [34].

In a study, the IC_{50} value of ADF extract against human hepatocellular carcinoma (HepG2) cells was 20.03 mg/ml after 24-hour exposure [31]. In comparison to the present study, the IC_{50} value of 8.96 mg/ml after 24-hour treatment period indicates that ADF extract was more effective against HSC-2 cells. The variation in the IC_{50} value could be due to many reasons. It could be the difference in proliferation rate of the cells or the cell density during the assay period [55].

On the contrary, the 50% inhibition of HSC-2 cells by ADP extract with an extremely low IC_{50} in the present study confirmed the anticancer property of ADP extract. In a similar study conducted by Thouri and colleagues in 2018, it was shown that date pit extract of two different varieties induced significant growth inhibition and apoptosis in a human cervical cell line (HeLa) and human liver cell line (HepG2) with an IC_{50} value of 0.028 mg/ml and 0.034 mg/ml, respectively. They also reported that the seed extract had no cytotoxic effects on normal fibroblast cell line [41]. Interestingly, polyphenols were distinguished by their low cytotoxic effects towards normal cell line and increased cytotoxicity towards cancer cell line [56].

The exact mode of action of ADF and ADP extract on HSC-2 cells is not fully understood. Nonetheless, a likely route of action for the extract could be via modulating oxidative stress and scavenging free radicals within the cells. Various stimuli including reactive oxygen species are known to activate HSC-2 cells [57]. In the present study, flow cytometry analysis indicated that the cytotoxic effects in HSC-2 cells were due to apoptosis induction. Furthermore, the results also displayed that with a lower concentration of both ADF and ADP extracts, HSC-2 cells were observed more in early apoptosis stage while late apoptosis stage was identified at a higher concentration of extracts. The data also

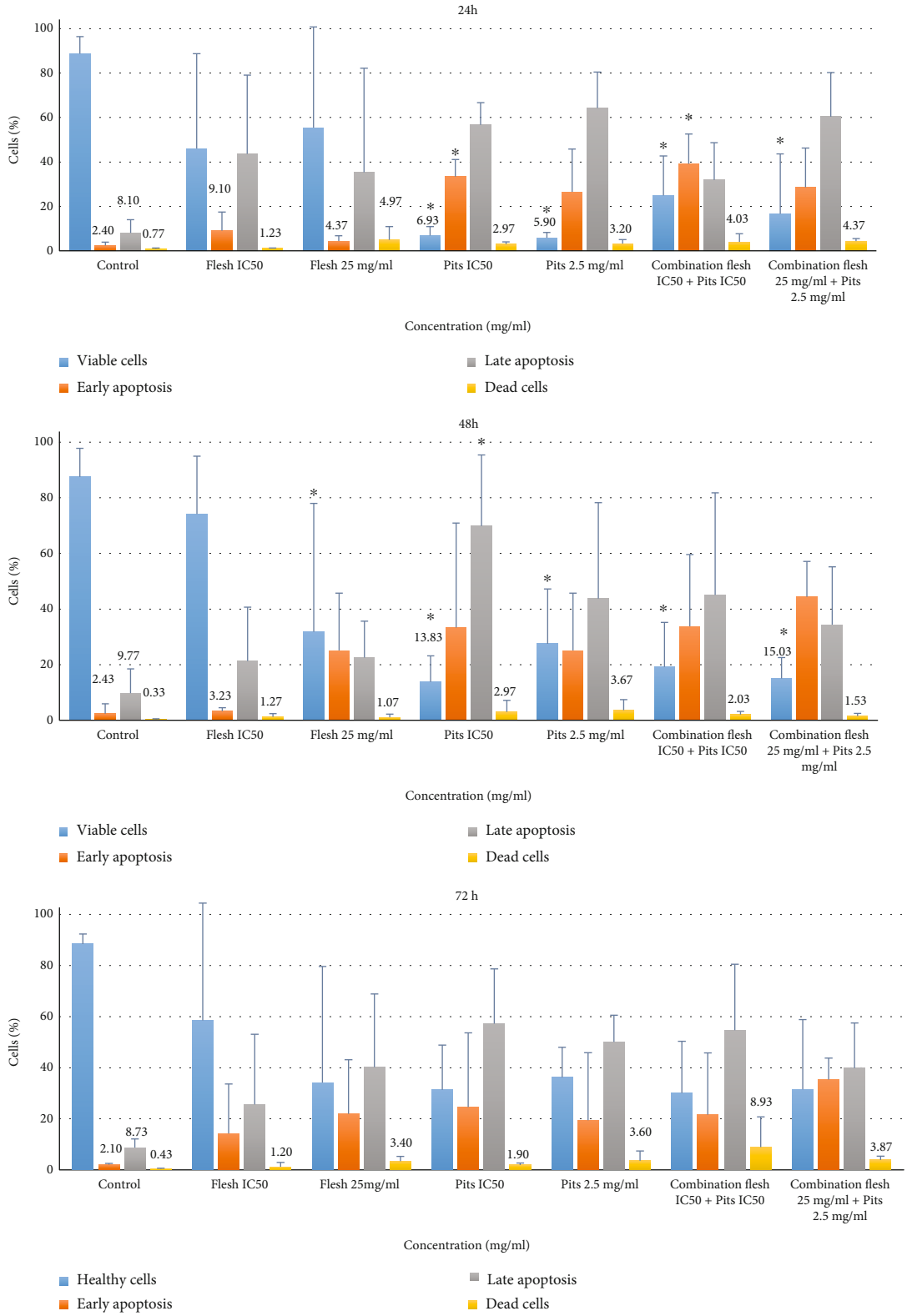
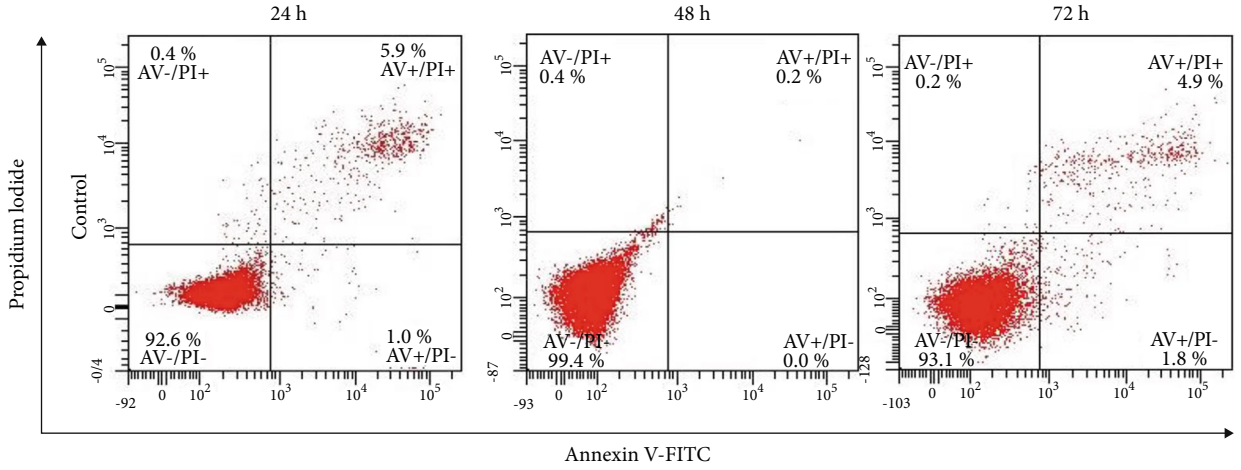
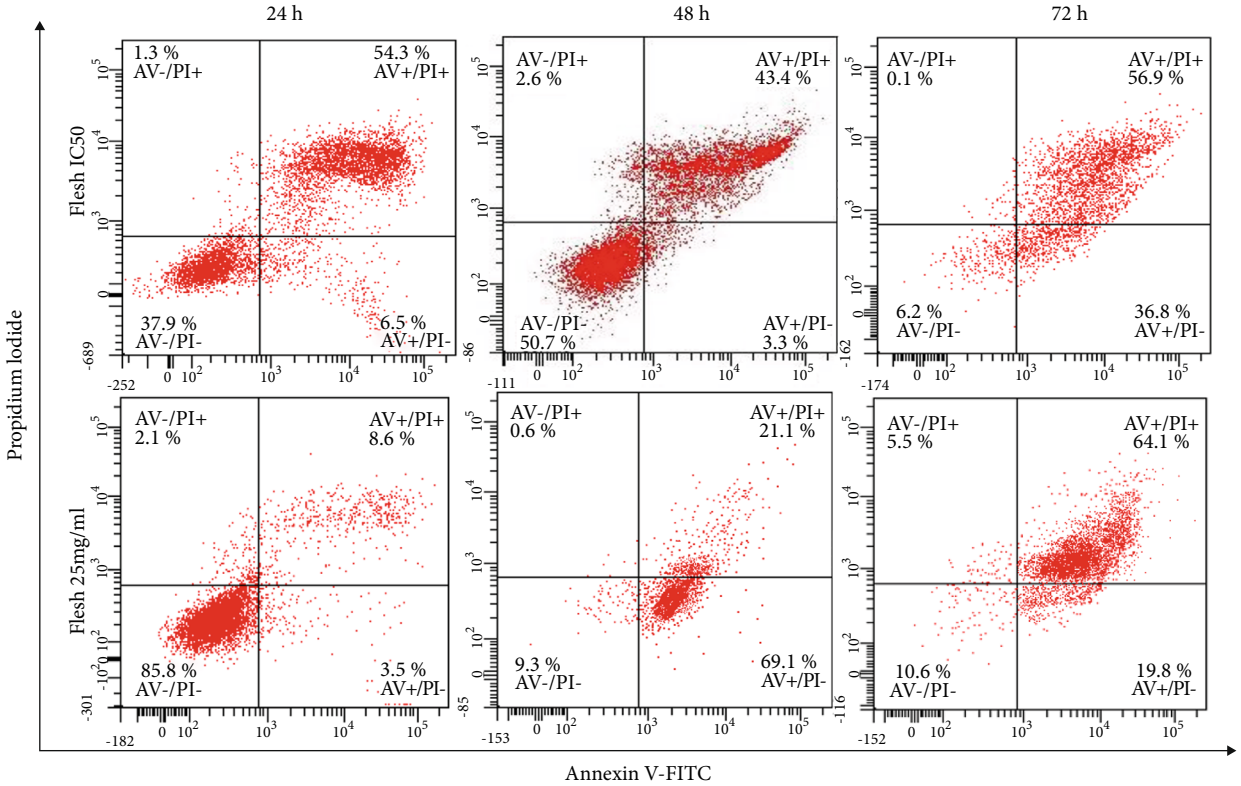


Figure 10: The effect of adding various concentrations of Ajwa date flesh and pit extracts on the apoptotic activity of HSC-2 cell line at 24 h, 48 h, and 72 h. The percentage of cells is shown in four stages, healthy cells, cells in early apoptotic stage, cells in late apoptotic stage, and dead or necrotic cells. The flesh IC50 value is 8.69 mg/ml, and pits IC50 value is 0.97 mg/ml. ∗ indicates that the treatment is significantly different from the control group at $p < 0.05$.

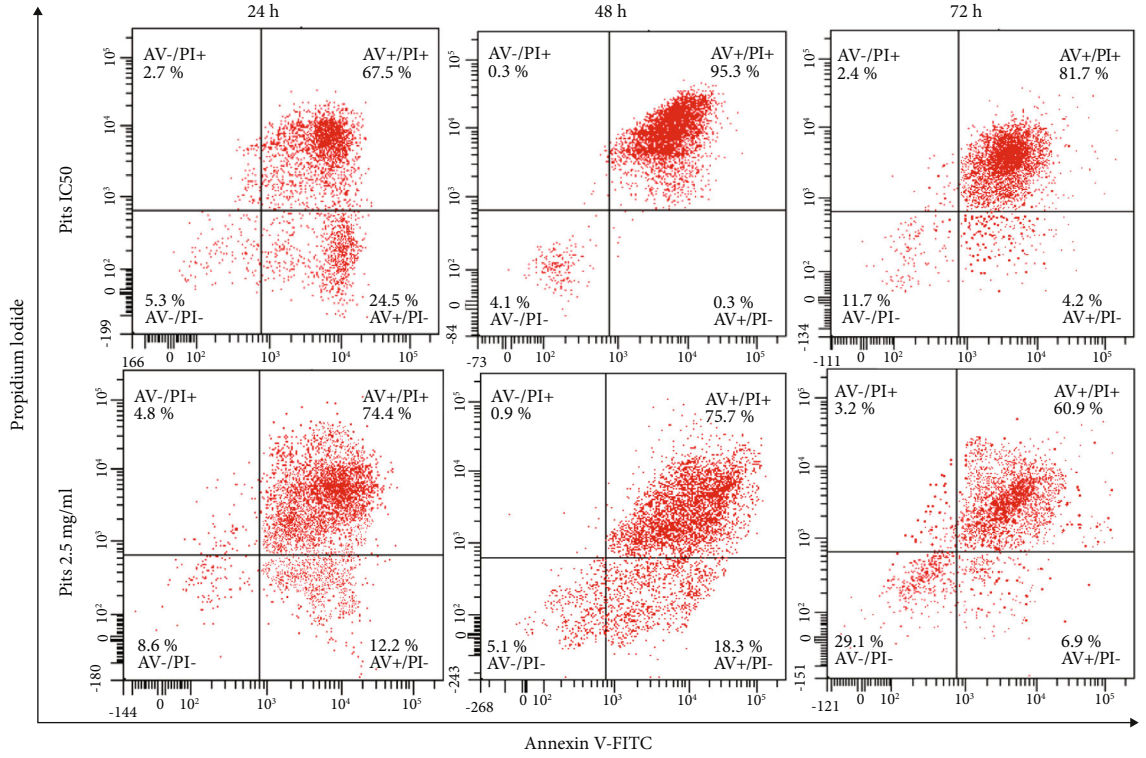


(a)



(b)

Figure 11: Continued.



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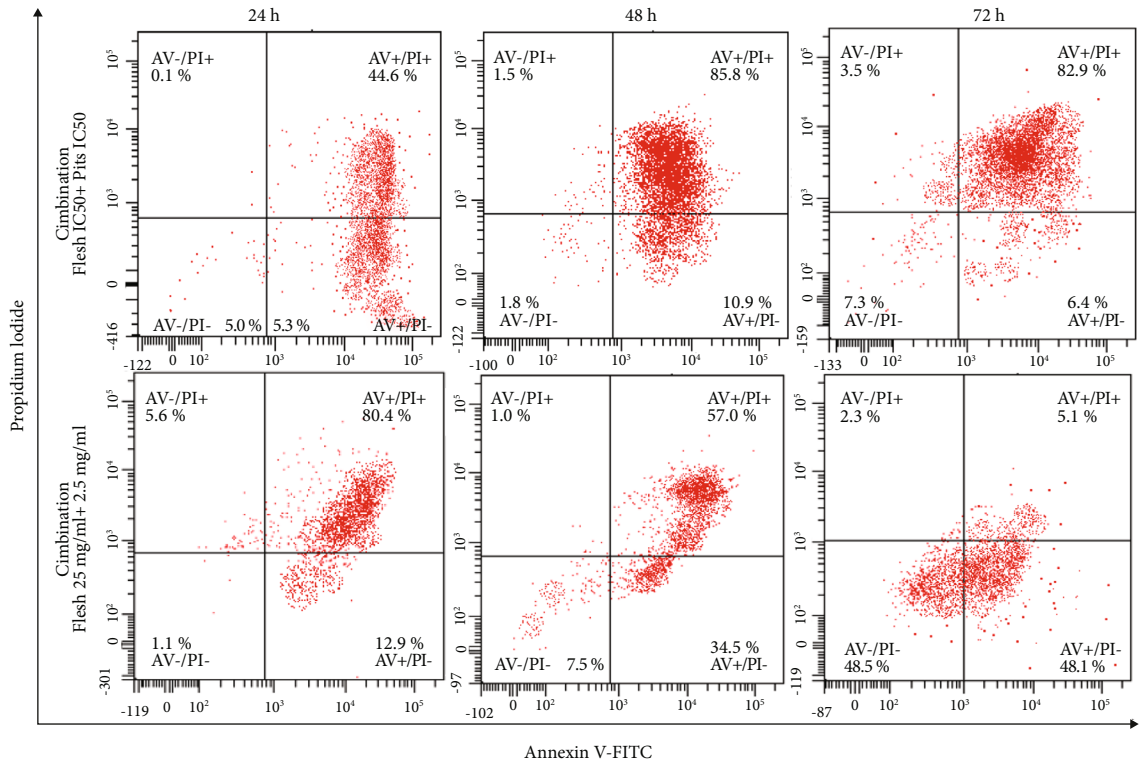

(d)

Figure 11: Annexin V-FITC and PI assays on HSC-2 cells treated with various concentrations of ADF and ADP for 24, 48, and 72 hours are represented in a dot-plot graph, where the AV-/PI- quadrant represents viable cells, AV+/PI- quadrant represents cells in early apoptosis, AV+/PI+ quadrant represents cells in late apoptosis, and AV-/PI+ quadrant represents dead or necrotic cells. (a) Represent the control group, (b) represent flesh with IC_{50} value and flesh with 25 mg/ml, (c) shows pits with IC_{50} and 2.5 mg/ml value, and (d) shows the combination of flesh IC_{50}+pits IC_{50} and flesh 25 mg/ml+pits 2.5 mg/ml.

suggests a time-dependent manner. The apoptotic activity exhibited by the ADF and ADP extracts may be attributed to the presence of phenols and flavonoid content like rutin, catechin, caffeic acid, apigenin, and quercetin which are present in Ajwa date flesh and pit [46, 58].

A previous study has reported the induction of apoptosis in breast cancer MCF-7 cells by the methanolic extract of Ajwa date flesh [27], in which it was reported that the percentage of total apoptotic cells was 68.1% at 25 mg/ml at 48 h of treatment duration, which correlates with the findings of present study where the total apoptotic cells at 48 h were 67.1% at 25 mg/ml of ADF extract. With the loss of cell membrane asymmetry, the phosphatidylserine (PS) flips towards the outside, which is considered to be a hallmark of cell in later stages of apoptosis [59]. Furthermore, ADP extract had increased apoptosis, compared to ADF. This result supports the results of MTT assay, in which there was significant cell death after the treatment of Ajwa date extract; apoptosis assay elaborates that the cell went through early and late stages of apoptosis before dying, thus confirming that Ajwa date induced apoptotic cell death in oral cancer cells.

The present study also investigated the combined effect of ADF and ADP extracts for the induction of apoptosis in HSC-2 cells. At 24 h, the percentage of early apoptotic cells with the combination treatment was 39.2% whereas when treated separately, it was 9.10% and 33.4% for ADF and ADP extract, respectively. Although it was not significantly higher than the single treatment, this may suggest that Ajwa date can induce higher level of apoptotic effect when used as a whole. Previously, many studies have evaluated the bioactive phenolic compounds of fruit seed more than the fruit flesh. Similar to the present study, grape seed extract had induced apoptotic cell death in OSCC [60]. Not only the seed but the peel (skin) and the edible part of grape have demonstrated apoptotic activity [61, 62]. Many other fruits have demonstrated higher polyphenol content in their seed than the edible flesh [47]. Therefore, it can be suggested that the fruit as a whole can be more beneficial in providing protection against carcinogenic effects and the seed part can be utilized in many different forms; like recently, date pit powder was used to make noncaffeinated coffee with coffee flavour [63].

5. Conclusion

In conclusion, the results from the present study suggest that Ajwa date (flesh and pit) demonstrated significant cytotoxic and antiproliferative activity against HSC-2 cells. The IC_{50} value demonstrated that Ajwa pit had a stronger antiproliferative effect than the flesh extract signifying its higher anticancerous potential, which was further confirmed in morphological alteration such as nuclear shrinkage, blebbing of cell membrane, and reduction in cell number, which are characteristic features of apoptotic cells. Furthermore, ADF and ADP extract was found to cause cell death via apoptosis in OSCC cells by flow cytometry assay in a dose- and time-dependent manner. The present study also demonstrates that Ajwa date as a whole can induce apoptosis more effectively than as separately. As research is still ongoing, further studies can be conducted to purify and identify individual components of the Ajwa date flesh and seed that are responsible for the anticancer properties and to better understand the in-depth molecular mechanism of action of apoptosis so that a novel chemotherapeutic drug can be made with less/no conventional side effects.

Data Availability

All data are available within the manuscript.

Conflicts of Interest

There are no conflicts of interest between the authors.

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Review Article

Traditional and Phytochemical Bases of Herbs, Shrubs, Climbers, and Trees from Ethiopia for Their Anticancer Response

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Ethiopia is one of Africa's six plant-rich countries, with around 60% of the plants being indigenous and most of them having medicinal properties. 80% of people in the country use these plants as a primary health care system to tackle different diseases, including cancer. This review is aimed at summarizing the evidence gained from diverse MPs in Ethiopia that have been used ethnobotanically and ethnopharmacologically for treatment of cancer. The primary data sources were Google Scholar, Web of Science, Science Direct, Scopus, PubMed, and other electronic scientific databases. This literature review showed that there are around 200 MPs used as anticancer. Seventy-four herbs, 39 trees, 77 shrubs, and 17 weed/climbers belonging to 56 families have been identified for their ethnobotanical anticancer potential, and 31 species were recognized for their pharmaceutically anticancer activities. The reviewed data also indicated that many Ethiopian MPs had been used to treat breast, lung, blood, and skin cancers and other tumors. Besides, the collected data showed that the leaves (36.76%), roots (27.2%), bark (12.5%), stem (5.1%), and fruit (7.35%) of plants are commonly used for the preparation of anticancer remedies. Among the reported plant species, Euphorbiaceae (10.71%), Acanthaceae (7.14%), and Asteraceae (7.1%) are the most prominent plant families being used to treat cancer ethnobotanically. Phytochemicals such as flavonoids (like xanthone, indirubin, flavopiridol, and silybin), alkaloids (like taxol, vincristine, evodiamine, and berberine), and physalin B, D, and F steroids exhibited anticancer activity on various cancer cell lines. The crude extracts of *Aerva javanica*, *Vernonia leopoldi*, *Withania somnifera*, *Kniphofia foliosa*, and *Catharanthus roseus* were powerful anticancer agents with an IC_{50} value below 10 μg/mL. Although several Ethiopian plants possess anticancer potential, only a limited number of plants are scientifically studied. Therefore, more scientific studies on anticancer MPs should be carried out; it may lead to discovering and isolating cost-effective and safe anticancer drugs.

1. Background

Plants are the sources of different chemical constituents such as essential oils, seed oils, and other phytochemicals, which gives a potential for various applications and pharmaceutical uses [1–4]. Specifically, traditional MPs (TMs) keep us healthy and treat different illnesses [5]. People have used TMs as primary health care contributors for thousands of years, and they play a vital role in preventing many diseases in resource-limited areas of developing countries [6, 7]. Nowadays, more attention has been given to TMs by different researchers because they can generate many uses and applications in the line of medicine and pharmacy [8]. In China, from total medicinal consumption, about 30% to 50% was gained from medicinal plants (MPs) [9]. In India, there are 17,500 native plant species from these 7,500 species that are used in ethnomedicines. About 2,000 aromatic and medicinal plant species are commercially used in Europe, while over 5,000 plant species are estimated to be used for medicinal purposes in Africa [10]. In Mali, Ghana, Nigeria, and Zambia, 60% of children's first treatment is obtained from a medicinal plant. In Ethiopia, approximately 80% of

the population uses MPs because of the cultural suitability for local medicine [9].

Ethiopia has a vast diversity of plant species due to the presence of various topographical settings, ranging from the highest mountain to a deep valley; as a result, Ethiopia is rich in MPs [11]. There are about 6,000 plant species in the country, with 12 percent of them being endemic [12]. In Ethiopia, more than 800 plant species have been claimed to treat more than 300 ailments [13]. The bioactive compounds are responsible for the pharmaceutical properties of MPs [14] and can be isolated from plant seeds, fruits, bark, leaves, stems, roots, and flowers [15]. Alkaloids, terpenoids, flavonoids, glycosides, and polyphenols are bioactive compounds obtained from MPs and are used to cure various diseases, including cancer [16].

Nowadays, cancer is one of the deadliest diseases in the world, which has been estimated to cause 9.9 million deaths in 2020 [17]. It also becomes a health problem in Ethiopia [18]. According to the report of Woldu et al. [19], each year, there are more than 150,000 cancer cases reported in the country; from these, about 4% result to deaths. The data obtained from the WHO indicated many types of cancers in Ethiopia; some of them are blood cancer, lung cancer, skin cancer, breast cancer, etc. [20]. Local people of Ethiopia have used different traditional practices to treat cancer [21]. MPs have been highly demanded in Ethiopia to treat cancer disease, because of their relatively low cost, the trust of communities on medicinal values of TMs being high, inadequate health centers, and shortage of drugs and personnel in clinics [13]. Some of the frequently cited anticancer MPs used by Ethiopian people to treat different types of cancers are *Aerva javanica*, *Bersama abyssinica*, *Asparagus africanus*, *Brucea antidysenterica* [22], *Nigella sativa* [23], *Matricaria chamomilla*, *Foeniculum vulgare* [24], *Zingiber officinale*, *Hibiscus sabdariffa*, *Glinus lotoides*, *Mentha piperita*, *Trachyspermum Ammi* [25], *Lepidium sativum* [26], *Commiphora myrrha* [27], *Ruta chalepensis* [28], and *Lippia adoensis* [29] as illustrated in Figure 1.

Although several Ethiopian plants were known to possess anticancer activity, very little scientific research is carried out on these MPs [30]. Also, a limited number of classes of secondary metabolites and pure isolated compounds were tested against cancer cell lines. Insufficient documentation on the ethnobotanical use of anticancer MPs is another problem in sharing traditional medicinal knowledge [31]. This review is aimed at giving an overview of the ethnomedicinal and phytochemical bases of anticancer MPs of Ethiopia.

2. Methods

Published research papers, review papers, proceedings, short communications, and book chapters on different MPs used to treat various forms of cancer in Ethiopia were retrieved from multiple databases such as PubMed, Web of Science, Scopus, and Google Scholar. More than 100 publications were obtained from 2007 to 2020. In the search process, keywords phytochemistry of anticancer plants of Ethiopia, traditional anticancer medicinal plant of Ethiopia, MPs used against cancer/tumor in Ethiopia, herbal medicine traditional medicine used against cancer in Ethiopia, and bioactive compounds isolated from the anticancer medicinal plant of Ethiopia were used. We classified the data according to the type of cancer (breast cancer, lung cancer, blood cancer, and skin cancer) and medicinal plant habits (shrub, herb, tree, weed, and climber). Additional important papers were also examined based on the reference list of the retrieved documents. ChemDraw was used to draw the structure of bioactive compounds, and pie charts were prepared using Excel software, while Endnote performed reference writing. We use the Natural Products Database for Africa (NDA) to write the botanical name and the local name of the medicinal plant.

3. Cancer Status in Ethiopia

Ethiopia is Africa's second-most populous country, and it is forecasted to become the world's ninth most populous country by 2050, with a projected increase in cancer burden [32]. Cancer is expected to account for around 5.8% of total national mortality in Ethiopia. Except for Addis Ababa, where population-based statistics are available, it is estimated that the annual incidence of cancer is about 60,960 cases and the annual mortality is over 44,000 [33]. According to a World Health Organization report on cancer [34], 77,352 new cancer cases were reported in both sexes of all ages, of which 26,754 were male of all ages and 50,598 were female of all ages. This showed that the number of new female cancer cases is 89.8% higher than that of males. Some of the recorded new cancer cases in 2020 were breast cancer (20.9%), leukemia (5.6%), cervix uteri (9.6%), colorectum (3.6%), and non-Hodgkin lymphoma (4.9%) (Table 1), and the mortality rates in the specified year were 24.1% (breast), 16.0% (cervix uteri), 3.9% (leukemia), 5.5% (ovary), 3.6% (lung), 3.4% (stomach), 5.5% (colorectum), and 5.9% (prostate) [34]. According to Tuasha et al. [11], from the total medicinal plant consumption used to treat cancer, 44.33% accounts for neck cancer, 14.0% breast cancer, and 10.67% skin cancer. The rest are the cancer of the brain, bone, rectal, lung, anus, cervix, and others.

3.1. Ethnobotanical Survey of MPs for Cancer Treatment. MPs are essential part of human life. For more than 2,000 years, they have been used as alternative medicine in the world [11]. Approximately 80% of these MPs globally are essential as the primary healthcare for fighting infections and treating illness [35]. MPs have been in continuous use over the years to manage cancer, particularly in most developing countries of the world [36]. The bioactive compounds of phytochemicals present in MPs are used to treat different diseases, including cancer [37]. For example, around 60% of drugs necessary for the cancer cure system have been derived from natural products of MPs [38]. Aromatic MPs are also crucial for medicinal purposes; they were considered the "father of medicine" by Hippocrates and ancient Greek physicians. Treating cancer and AIDS/HIV are their main benefits [21].

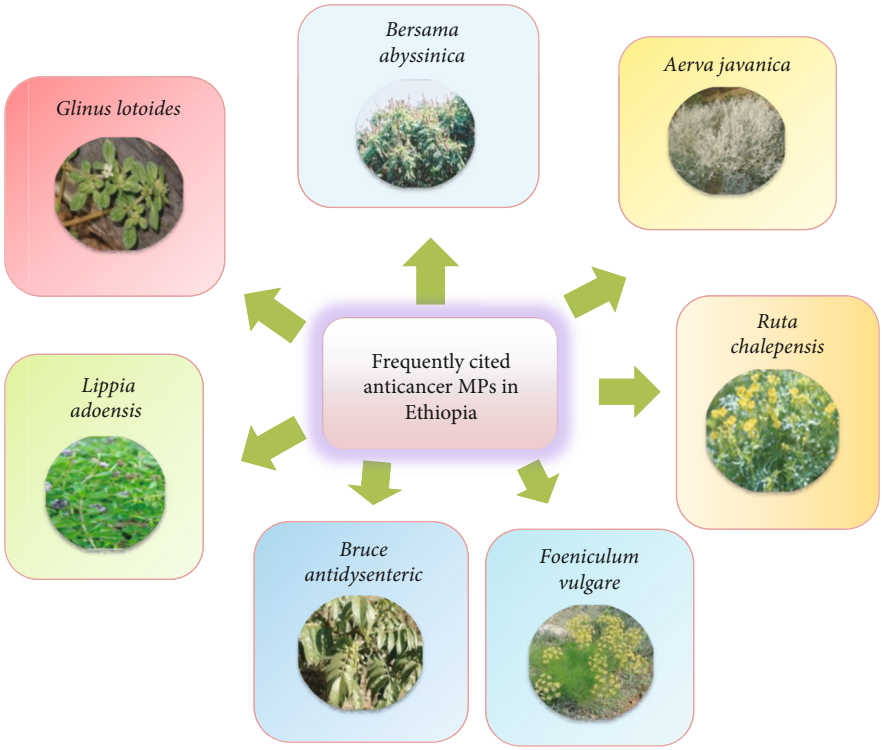


Figure 1: Frequently cited anticancer MPs found in Ethiopia.

Table 1: Number of new cancer cases in 2020 in Ethiopia in number and percentage [34].

New cancer cases	Both sexes of all ages	Males of all ages	Females of all ages
Non-Hodgkin lymphoma	3,824 (4.9%)	2,359 (8.8%`)	1,465 (2.9%)
Leukemia	4,361 (5.6%)	2,565 (9.6%)	1,796 (3.55%)
Cervix uteri	7,455 (9.6%)	—	7,455 (14.7%)
Breast	16,133 (20.85%)	—	16,133 (31.9%)
Colorectum	6,048 (7.8%)	3,121 (11.7%)	2,927 (5.8%)
Prostate	2,720 (3.51%)	2,720 (10.2%)	—
Ovary	2,655 (3.43%)	—	2,655 (5.2%)
Other cancers	34,156 (44.15%)	15,989 (59.76%)	18,167 (35.9%)
Total	77,352	26,754	50,598

Many medicinal plant species found in Ethiopia are used to treat different types of illnesses for many years. Because the society believes in the therapeutic value of traditional medicines, of health center insufficiency, of the relatively low costs, of culturally related traditions, and of shortages of clinics and medical personnel, they are very popular in Ethiopia [13]. In Ethiopia, a large number of the human population (80%) and livestock (90%) directly or indirectly depend on traditional medicine [39]. According to a study conducted on traditional MPs in Ethiopia, the frequently cited diseases treated by these plants were cancers/tumors, stomach aches, wounds, coughs, headaches, skin diseases, toothaches, and diarrhoea [13]. Different studies on the ethnobotanical use of MPs from other parts of the country showed that traditional MPs are widely practiced to treat various cancer diseases such as lung cancer, breast cancer, and skin cancer [11]. Because of its ease of access and cultural acceptance, cancer patients choose traditional MPs for therapeutic approaches [40]. Ethnobotanical practices to treat cancer in Ethiopia are listed in Table 2.

Table 2 shows the list of 200 MPs which are used ethnobotanically against anticancer. Out of these, 33.8% are herbs, 17.9% trees, 39.5% shrubs, and 8.8% weed/climbers. Among the 56 families, Euphorbiaceae (10.71%), Acanthaceae (7.14%), and Asteraceae (7.1%) are the most prominent families which are used for ethnobotanical anticancer preparation. Regarding their distribution, 24% of MPs were found in Southern Nations, Nationalities, and People (SNNP), 21% in the Oromia region, and 20% in the Amhara regional state, as shown in Figure 2. The reviewed data also indicated that many Ethiopian MPs had been used to treat breast, lung, blood, and skin cancers. Plant sections that are widely

Table 2: Ethnobotanical uses of anticancer plants in Ethiopia.

Family	Habitat	Plant name	Local name	The region in which the plant can be found	Part of the plant used to treat cancer	Type of cancer	Ethnobotanical preparation	Reference
Acanthaceae	H	*Blepharis maderaspatensis*	Boke	Harari region	Roots	Breast cancer	The root is powdered and boiled with coffee used to like tea	[21]
Acanthaceae	S	*Justicia schimperiana*	Kitkit	North bench in SNR	Roots	Lung cancer	Until eating, fresh roots are crushed and boiled and the cool decoction is drank	[40]
Aloaceae	S	*Aloe pirottae*	It	Sidama zone in SNNP	Leaves	NSC	A spoonful of the plant's pulp or leaf is mixed with honey and consumed twice a day	[11]
Aloaceae	S	Aloe sp.	Gurtawaqota	Mizan Aman in SNMP	Leaves	NSC	The sap, which is made from the fresh root, is applied to the affected area	[30]
Amaranthaceae	H	*Aerva javanica*	Tobia	Dek Island in Amhara region	Roots	Breast cancer	The plant root is powdered and combined with the bat's blood and given orally before breakfast in the morning	[41]
Amaranthaceae	H	*Pupalia lappacea*	Kent-omme	Harari region	Leaves	NSC	The leaves are boiled and pasted with edible oil and orally taken	[21]
Amaranthaceae	W	*Achyranthes aspera*	Koch-ashite	Mizan Aman in SNMP	Leaves	NSC	Animal butter mixed with leaves of the plant roasted on a metal plate, crushed into powder, and then applied on the affected part	[30]
Amaryllidaceae	H	*Scadoxus multiflorus*	Dem-astefit	Mizan Aman in SNMP	Roots	NSC	Combination with other herbs and applied topically	[11]
Amaryllidaceae	H	*Crinum abyssinicum*	Yegibb shinkurt	Dek Island in Amhara region	Leaves	NSC	The leaf powder is administered topically to the afflicted area, together with hyena excrement and latex, to provide progressive relief	[42]
Anacardiaceae	T	*Ozora insignis*	Rukeylu, Garri	Harari region	Root	NSC	The plant's root has been crushed, and the bandage has been placed over it	[21]
Apiaceae	H	*Centella asiatica*	Goro-ngoc	Sheko in SNNPR	Leaves	NSC	The young leaves of the plant are powdered, and the sap is sniffed	[30]
Apiaceae	H	*Foeniculum vulgare*	Ensilal	East Gojjam in Amhara region	Roots	Lung cancer	The roots of the plant are mixed with other herbs used and taken orally	[30]
Apiaceae	H	*Hydrocotyle mannii*	Yeti-medhanit	North bench in SNNPR	Leaves	NSC	The leaves of the plant at a young age are powdered and put in the affected area	[30]
Apiaceae	H	*Ferula communis*	Dog	Libo-Kemkem in Amhara region	Root	Lung cancer	Fresh root crushed and drank with water orally	[43]
Apocynaceae	H	*Echidnopsis dammaniana*	Murali	Gene in Afar region	Stem	NSC	The stems of the plant are cut and the sap is introduced on the area of the affected part	[40]
Apocynaceae	H	*Catharanthus roseus*	Wulu-wusha	Dawro in SNNPR	Aerial part	NSC	Liver infection, wounds, and rheumatism	[44]
Apocynaceae	H	*Huernia macrocarpa*	Yemidirkulkual	In all the Amhara region	Latex	Skin cancer	The wound part is treated by inserting the mixture of the latex of the plant with "sumanfar"	[45]

Table 2: Continued.

Family	Habitat	Plant name	Local name	The region in which the plant can be found	Part of the plant used to treat cancer	Type of cancer	Ethnobotanical preparation	Reference
Apocynaceae	S	*Carissa spinarum*	Agam	Bahir Dar Zuria in Amhara region	Twigs and leaves	Oral cancer	Honey mixed with a paste made by crushing the young twig and fresh leaf of the plant. The mixture is given orally until a cure	[46]
Apocynaceae	S	*Calotropis procera*	Kobo	Gene, in Afar region	Flower and root	NSC	The sap is added to the region after fresh roots are crushed	[47, 40]
Apocynaceae	C	*Pentarrhinum insipidum*	Barohula	Gewane in Afar region	Root	NSC	The plant's fresh roots are pounded, and the sap is added to the affected region	[30]
Asclepiadaceae	H	*Caralluma speciosa*	Ya'iibera	Harari region	Stem	NSC	The stem of the plant is crushed and bandaged in the affected part	[21]
Asclepiadaceae	S	*Kanahia laniflora*	August	Around West Gojjam in Amhara region	Leaves and latex	NSC	Fresh leaf juice with latex is taken orally or applied topically	[11]
Asparagaceae	C/W	*Asparagus africanus*	Sarita, hidden saree	Harari region in eastern Ethiopia	Root	Breast cancer	The concoction taken orally to treat tumors	[21]
Asparagaceae	C/W	*Asparagus africanus*	Yes-kest	Zegie Peninsula in Amhara regional state	Roots	Uterine cancer and breast cancer	The root is pounded, boiled, and drank	[48]
Asparagaceae	C/W	*Asparagus africanus*	Yes-kest	Kembatta Tembar in SNNPR	Root	Breast cancer	Used to treat uterine prolapse and breast tumours, among other things	[38]
Asphodelaceae	H	*Kniphofia foliosa*	Shushube	Bale Gobain Oromia region	Root	NSC	The dried roots are crushed and powdered and mixed with honey	[30]
Aspleniaceae	S	*Artemisia absinthium*	Ariti	None	Leaves	NSC	The leaves of the plant are mixed with *Zingiber officinale* and *Ruta chalepensis*, made into an infusion, filtered, and drank	[30]
Asteraceae	H	*Bidens macroptera*	Adey Abeba	Libo kemke district in Amhara region	Flower	Brain cancer	The powdered flower part is used	[43]
Asteraceae	H	*Bidens macroptera*	Adey Abeba	Libo Kemke in Amhara region	Leaves	NSC	The leaves are dried and powdered and applied to the area which needs to be cured	[43]
Asteraceae	H	*Artemisia absinthium*	Natura	Sodo Zuria in SNNP	Leaves	NSC	Dried leaves of the plant are powdered and macerated in coffee or tea	[30]
Asteraceae	H	*Artemisia afra*	Agfa	Doyo Gena in SNNPR	Leaves	NSC	Juice squeezed and taken orally	[30]
Asteraceae	H	*Cineraria abyssinica*	Item-firh	Bale Robe in Oromia region	Leaf and aerial parts	NSC	The aqueous decoction of the aerial and leaf parts of the plant is taken orally	[48]
Asteraceae	T	*Bacchae-oides filigera*	Weynagift		Leaves	NSC	Decocted leaf is drank to recover from lines	[30]

Table 2: Continued.

Family	Habitat	Plant name	Local name	The region in which the plant can be found	Part of the plant used to treat cancer	Type of cancer	Ethnobotanical preparation	Reference
				Nekem and Jimma in Oromia region				
Asteraceae	H	*Artemisia annua*	Artemisia	Sodo Zuria in Sidama regional state	Leaves	NSC	Dried leaves are powdered and decocted in hot water and taken	[30]
Asteraceae	T	*Vernonia auriculifera*	Barawa	Doyo Gena	Leaves	Skin cancer	The leaves of the plant in a fresh state are grounded, and the sap is applied to it	[22]
Asteraceae	T	*Vernonia auriculifera*	Reji	Wondo Genet in SNNP	Leaves	Skin cancer	The plant's leaves in a fresh state are grounded, and the sap is applied to it	[22]
Asteraceae	S	*Echinops jericho*	Kericho	Harari region	Root	NSC	Powdered with *Hydnora johannis* tuber and added in the food that we eat	[21]
Asteraceae	H	*Guizotia scabra*	Sheshota	Doyo Gena in SNNPR	Leaves	NSC	The sap from fresh leaves is added to the affected area after they have been crushed	[30]
Asteraceae	S	*Vernonia auriculifera*	Barawa	SNMP	Leaves	NSC	The plant's fresh leaves are crushed, and the sap is added	[30]
Asteraceae	S	*Solanecio gigas*	Ababa	Doyo Gena in SNNPR	Leaves	NSC	The sap, made from fresh leaves of the plant, is crushed, and the sap is applied	[30]
Asteraceae	W	*Plectocephalus varians*	Este-Yohannes	Around West Gojjam in Amhara region	Whole part	NSC	The entire fresh plant is squeezed and applied	[11]
Asteraceae	S	*Vernonia amygdalina*	Grawa	Sidama regional state	Shoot	NSC	Tender shoots are pounded and soaked with water and given to the patient	[13]
Boraginaceae	T	*Cordia africana*	Size	South Gonder in Amhara region	Leaves	NS	The juice is made from the leaves of the plant and its paste is used to treat cancer	[46]
Boraginaceae	T	*Ehretia cymosa*	Makeba	Across the region of Ethiopia	Bark	Nectar and anal cancer	The root bark is applied topically in conjunction with other herbs	[11]
Brassicaceae	H	*Brassica carinata*	Gome-nzer	Debark district in Amhara region	Seed	Skin cancer	Seed of the plant with leaf and seed of the plant alone are crushed, powdered, and mixed with honey and creamed on the affected area	[49]
Capparidaceae	H	*Cleome brachycarpa*	Berbere	Gene in Afar regional state	Leaves	NSC	Fresh leaves of the plant are grounded, and the sap is placed on the affected part	[30]
Capparidaceae	T	*Boscia senegalensis*	None	None	Root	NSC	By pounding and powdering given orally	[50]
Capparidaceae	S	*Canada farinosa*	Qala-anqaal	Yalo district, zone 4 in Afar region	Leaves	Breast cancer	Not found	[51]

Table 2: Continued.

Family	Habitat	Plant name	Local name	The region in which the plant can be found	Part of the plant used to treat cancer	Type of cancer	Ethnobotanical preparation	Reference
Celastraceae	S/T	*Maytenus senegalensis*	Kebkeb	Gondar Zuria district in Amhara region	Bark	NSC	The plant's bark is crushed, boiled, and filtered, and one cup is served	[52]
Celastraceae	S	*Maytenus ovatus*	Not specified	NA	Leaf	NSC	The plant's leaf paste, mixed with honey, is taken orally every morning and evening before it heals	[53]
Celastraceae	S	*Gymnosporia buchanan*	Atat	Gondar in Amhara region	Leaves	NSC	Crushed leaves are mixed with honey to produce a paste	[54]
Celastraceae	S	*Maytenus senegalensis*	Atat	Gondar Zuria district, Amhara region	Leaves	NSC	It is applied to the affected region with a paste	[52]
Combretaceae	T	*Lagenaria siceraria*	Basubaaqula	Dega Damot district/Amhara region	Fruit	Breast cancer	The leaves of the plant are powdered, squeezed, and put on the affected area (wound)	[55]
Combretaceae	T	*Combretum collinum*	Abalo	Debark district, North Gondar zone in Amhara region	Leaves	NSC	The leaves of the plant are grounded, crushed, and put on the wound or tumour	[11]
Commelinaceae	H	*Commelina benghalensis*	Value-cha	Doyo Gena in SNMP	Roots	NSC	Fresh roots of the plant are dried and pounded, and the sap is put on the affected part	[30]
Convolvulaceae	S	*Ipomoea marmorata*	Gumna-kul	Harari region in eastern Ethiopia	Root	NSC	The new tuber is consumed, and a concoction is taken orally	[21]
Crassulaceae	H	*Kalanchoe petition*	Inda-hula	Bale in Oromia region	Leaves	Breast cancer and skin cancer	The plant leaves, fresh, are soaked for two minutes and put on the affected part. The plant is powder and mixed with hyena faces and latex	[22]
Crassulaceae	H	*Kalanchoe lanceolata*	Bose	Nekemte in Oromia region	Leaves and roots	NSC	The juice which is made from the fresh root and leaves is squeezed and drank	[56]
Cucurbitaceae	H	*Cucumis prophetarum*	Yemdirembuay	Debre Libano in Oromia region	Roots	Skin cancer	The root of the plant is dried and powdered and, when combined with water, given orally	[42]
Cucurbitaceae	H	*Clutia abyssinica*	File-fej	Across the region of Ethiopia	Whole part	NSC	The whole part of the plant, together with *Coffea robusta* and *Coffea richardiana*, is used topically	[11]
Cucurbitaceae	H	*Momordica friesiorum*	Wof tech	Across the region of Ethiopia	Roots	NSC	The root of the plant is combined with other herbs and applied topically	[11]
Cucurbitaceae	T	*Croton macrostachyus*	Bisana	Hawassa, Sidama regional state	Leaves	Skin cancer and wound cancer	The juice of the leaves of the plant and its paste are applied on wound cancer, and crushed and powdered fresh leaves are used on the affected part	[46, 30]

Table 2: Continued.

Family	Habitat	Plant name	Local name	The region in which the plant can be found	Part of the plant used to treat cancer	Type of cancer	Ethnobotanical preparation	Reference
Cucurbitaceae	W/C	*Lagenaria siceraria*	Qil	Hawassa city/ Sidama regional state	Root	NSC	The root of the plant is pounded, powdered, and drank	[39]
Cucurbitaceae	W/C	*Lagenaria siceraria*	Qil	None	Leaves	NSC	Crushed and squeezed leaves are applied to the infected area to alleviate cancerous sores	[57]
Euphorbiaceae	T	*Euphorbia tirucalli*	Kinc hib	South Wollo in Amhara regional state	Latex of roots	Skin cancer	The fresh sap/latex of the plant is collected and creamed all over the body. Latex is given for topical application	[58]
Euphorbiaceae	H	*Euphorbia platyphyllos*	Anitrfa	Mecha district in Amhara region	Latex	NSC	Fresh latex of the plant is put topically on the tumour	[22]
Euphorbiaceae	H	*Euphorbia lathyris*	Hada-amii	Chelya district in Oromia region	Stem	Breast cancer	Steam of the plant is chopped and fumigated to the affected breast	[22]
Euphorbiaceae	T	*Euphorbia abyssinica*	Cultural	Around Debre Libanos in Oromia region	Latex	Skin cancer	Latex is eaten with teff powder of the plant bread or takes the latex and then painted on the spot	[45]
Euphorbiaceae	T	*Acacia oerfota*	Seraw	Yalo district zone 4 in Arar region	Leaves	Breast cancer	The leaf of the plant is crushed and put nasally and topically	[11]
Euphorbiaceae	T	*Euphorbia abyssinica*	Qulqwal	Debre Libanos in Oromia region	Latex, stem, and bark	Skin cancer	Decoction and placing of the latex to the affected part; and the paste of the bark and stem is rub to the affected area	[42]
Euphorbiaceae	T	*Euphorbia tirucalli*	Kinship	Dale district in Sidama regional state	Bark	Skin cancer	Latex is combined with powder made from beans given to eat after food, and latex is dropped on the affected part to treat skin cancer	[58]
Euphorbiaceae	T	*Euphorbia tirucalli*	Kinship	Fiche in Oromia region	Latex and root	Skin and neck cancers	Eaten and added to the skin after being mixed with bean powder	[59]
Euphorbiaceae	T	*Erythrina brucei*	Kiara/Woolens	Dale district in Sidama region	Bark	NSC	The juice made from the bark of the plant is drank for the treatment of cancer	[46]
Euphorbiaceae	T	*Croton macrostachyus*	Bisana	Hawass in Sidama region	Leaves and seeds	NSC	The leaves or the seed of the plant are crushed and inserted into the wound	[45]
Euphorbiaceae	S	*Ricinus communis*	Qenbo'o	Hawassa in Sidama region	Root	Breast cancer	The root of the plant is chewed and swallowed or applied to the affected part	[30]
Euphorbiaceae	S	*Colutea abyssinica*	Graduate	Across the region of Ethiopia	Root and seed	Cervical and rectal cancer	The root and seed are mixed with other herbs and given topically	[11]
Euphorbiaceae	S	*Jatropha curcas*	Ayderke	NA	Seed	NSC	Tumours are treated with a paste made from the plant seed powder mixed with honey	[44]

Table 2: Continued.

Family	Habitat	Plant name	Local name	The region in which the plant can be found	Part of the plant used to treat cancer	Type of cancer	Ethnobotanical preparation	Reference
Euphorbiaceae	T	*Acalypha acrogyna*	Gullo	Gondar in Amhara region	Leaves	NSC	The leaves of the plant are crushed and combined with honey	[60]
Euphorbiaceae	T	*Acalypha acrogyna*	Gullo	Gondar in Amhara region	Leaves	NSC	In the morning, a mixture of honey and paste made from the leaves of the plant is given orally and heated leaves are applied externally over the affected area	[42]
Euphorbiaceae	S	*Senna alexandrina*	Mekerbaa	NA	Bark	NSC	The powdered bark of the plant is creamed on the swelling	[11]
Euphorbiaceae	S	*Euphorbia schimperiana*	Gendal-elata	Doyo Gena in SNNPR	Root	NSC	The plant's fresh roots are crushed, and the sap is added to the affected area	[13]
Euphorbiaceae	S	*Euphorbia polyacantha*	Carrico	NA	Latex	Skin cancer	The latex of the plant is squeezed and creamed on the affected part	[42]
Euphorbiaceae	S	*Calpurnia aurea*	Digita	Debre Libanos monastery in Oromia region	Leaves	Neck cancer	The leaves of the plant are powdered and soaked in cold water and taken orally	[22, 42]
Euphorbiaceae	S	*Senna singueana*	Busha	Across the region	Leaves and bark	NSC	The powdered leaves of the plant are applied topically	[30]
Euphorbiaceae	S	*Dichrostachys cinerea*	Ader	Yalo district in Afar region	Root	Skin cancer	The root of the plant is pounded and given orally	[51]
Fabaceae	T	*Acacia seyal*	Wacho	Bensa in SNMP	Leaves	NSC	The leaves of the plant are chewed and swallowed	[39]
Fabaceae	T	*Albizia lebbeck*	NA	Adekfurdu in Tigray region	Root	NSC	Wheat dough paste of root powder is applied on the affected part	[61]
Fabaceae	S	*Melilotus suaveolens*	Egg	Gubalafto district in northern Ethiopia	Leaves	Lung cancer	Crush, smash in water, filter, and then drink	[45]
Fabaceae	S	*Calpurnia aurea*	Vegeta	Debre Libanos in Oromia region	Leaves	Neck cancer	Powder is mixed with water and taken orally	[59]
Fabaceae	S	*Calpurnia aurea*	Digita	Debre Libanos in Oromia region	Leaves	Neck cancer	The leaves of the plant are made a paste and put on the affected area	[22]
Fabaceae	S	*Calpurnia aurea*	Digita	Bahir Dar Zuria in Amhara region	Leaves and seed	NSC	Powdered leaves or seeds are immersed in cold water and then drank	[42]
Fabaceae	S	*Crotalaria incana*	Chelke	Doyo Gena in SNNPR	Leaves	NSC	Fresh leaves are pounded and the sap was put on the affected area	[30]
Fabaceae	S	*Senna singueana*	Gefa	Bahir Dar Zuria in Amhara region	Leaves	NSC	Fresh leaves are pounded, soaked in water, and drank	[30]

Table 2: Continued.

Family	Habitat	Plant name	Local name	The region in which the plant can be found	Part of the plant used to treat cancer	Type of cancer	Ethnobotanical preparation	Reference
Fabaceae	S	*Crotalaria agatiflora*	Unknown	Bale Goba in Oromia region	Seed	NSC	Dry seeds are powdered, mixed with honey, and put on the affected area	[30]
Fabaceae	T	*Millettia ferruginea*	Henge-ddicho	Sidama regional state	Bark	NSC	The juice of bark is drank for cancer treatment	[46]
Flacourtiaceae	S	*Dovyalis abyssinica*	Kashim	Fiche district in Oromia region	Fruit	NSC	Eating 6–10 fruits per day	[58]
Flacourtiaceae	S	*Dovyalis abyssinica*	Kashim	Dale district in SNMP	Bark	NSC	The raw bark of the plant was chewed and then consumed	[58]
Hydnoraceae	T	*Hydra abyssinica*	Shifa'-a weyn	Harari region	Bark and roots	NSC	Bark or root of the plant is powdered with *Echinopskebericho* tuber and added in the daily food and eating	[21]
Iridaceae	H	*Gladiolus candidus*	Milas-golgul	Dega Damon in Amhara region	Roots	NSC	The root is dried and powdered and put on the affected area or drank	[57]
Iridaceae	H	*Gladiolus candidus*	Milas-golgul	Dega Damot in Amhara region	Roots	NSC	The plant's root is dried, crushed, and put on the wound part, or root powder is taken orally with water	[57]
Juncaceae	H	*Juncus effusus*	Etse-felatsut	Across the region of Ethiopia	Roots	NSC	The root of the plant is used by mixing with other herbal plants and applied topically on the affected area	[11]
Juncaceae	H	*Cleroden-drum myricoides*	Misrichi	Dale district in SNNP	Leaves	Blood cancer	The honey is mixed with the grounded leaf part of the plant and drank, or the root of the plant is boiled and mixed with *Zanthoxylum chalybeum* shoot	[49]
Lamiaceae	H	*Leonotis ocymifolia*	Arma-USA	Bale Goba in Oromia region	Leaves	NSC	Fresh leaves are crushed, macerated overnight, and drank	[30]
Lamiaceae	H	*Ajuga leucantha*	Tiksasht	North Bench in SNMP	Leaves	NSC	The fresh leaves of the plant are grounded, and the sap is put on the affected area	[30]
Lamiaceae	H	*Ocimum gratissimum*	Make-desisa	Wendo Genet in SNMP	Roots	NSC	Fresh roots are crushed, boiled, and drank	[30]
Lamiaceae	H	*Salvia nilotica*	Keskeo	North Bench in SNNP and Gonde in Amhara region	Leaves	NSC	The fresh leaves of the plant are powdered with water and made a paste	[60]
Lamiaceae	H	*Thymus schimperi*	Design	Bale Goba in Oromia region	Leaves	NSC	Dry leaves are decocted and drank	[30]
Lamiaceae	S	*Premna schimperi*	Xullangee	Bule Horra in Oromia region	Leaves	NSC	Pounding and making solution	[5]
Lamiaceae	T	*Pycnostachys abyssinica*	Montana	Doyo Gena in SNNPR	Leaves	NSC	The sap is added to the affected area by crushing the pounded fresh leaves of the plant	[30]

Table 2: Continued.

Family	Habitat	Plant name	Local name	The region in which the plant can be found	Part of the plant used to treat cancer	Type of cancer	Ethnobotanical preparation	Reference
Lamiaceae	S	*Leonotis ocymifolia*	Ye fereszeng	Fiche district in Oromia region	Leaves	Neck cancer	For one day, the chopped leaves of the plant are applied to the affected area	[62]
Lamiaceae	S	*Leonotis ocymifolia*	Raskimir	Across the region of Ethiopia	Root	NSC	Sometimes, it is used with the combination of *Leonotis africana*	[11]
Lamiaceae	S	*Satureja abyssinica*	Este meaza	Across the region of Ethiopia	Leaves	NSC	The leaves of the plant are combined with other herbs and applied topically	[11]
Lamiaceae	S	*Roca myricoides*	Mardhisiis a	Bule Hora, Oromia region, Bensa in SNMP	Leaves and root	NSC	Crush the root, mix it with butter, and apply; chop the leaf and eat or apply	[30, 49]
Liliaceae	H	*Gloriosa superba*	Etse-lebona	In most of Ethiopia and the Harari region	Roots, seeds, and leaves	Breast cancer	Seeds and roots of the plant dried and crushed and mixed with water are taken orally. The root of the plant is chewed and put externally on the affected breast. The leaves of the plant are made paste and tied on the tumour	[21, 42]
Lobeliaceae	T	*Lobelia rhynchopetalum*	Etse-kemun	Across the region of the country	Root	NSC	The root of the plant is combined with other herbs and put topically	[45]
Loganiaceae	S	*Buddleja polystichum*	Anfar	Dale district in SNNP	Leaf	NSC	Crushed, cold macerated, and taken orally	[49]
Loganiaceae	W/C	*Malva verticillata*	Lut	Ada's district, east Shewa zone in Oromia region	Leaves	NSC	The leaf is crushed and attached to the swelling after being warmed over an open flame	[13]
Malvaceae	S	*Sida schimperiana*	Kotejebessa	Wendo Genet in SNMP	Root and leaves	Wound cancer	Fresh leaves and roots of the plant are pounded, macerated, and drank	[30]
Malvaceae	S	*Sida schimperiana*	Chef Greg	Nekemte town, east Wellega in Oromia region	Root	Breast cancer	The juice made from fresh root is mixed with honey and taken orally	[30]
Malvaceae	S	*Sida schimperiana*	Chef Greg	Debark district in Amhara region	Leaves and root	Neck cancer	The root and leaves of the plant are crushed, powdered, and then put on the affected part	[11]
Malvaceae	H	*Malva verticillata*	Lut	East Shewa zone in Oromia region	Leaves	Neck cancer	The leaf of the plant is crushed, warmed, and then tied on the swelling	[13]
Meliaceae	T	*Lepidotrichilia volkensii*	Tabecho	Bensa in SNNP	Leaves and fruit	NSC	The leaves and fruit of the plant are chopped and mixed with water and taken orally	[63]

Table 2: Continued.

Family	Habitat	Plant name	Local name	The region in which the plant can be found	Part of the plant used to treat cancer	Type of cancer	Ethnobotanical preparation	Reference
Melianthaceae	T	*Bersama abyssinica*	Azamirr	Bahir Dar Zuria in Amhara region	Bark	NSC	The plant's bark or stem is used to make an injection used to treat some types of tumours	[22]
Melianthaceae	T	*Bersama abyssinica*	Azamir	Dale district in Sidama region	Bark	NSC	The bark of the plant is crushed and boiled, and then a small amount is drank	[30]
Menispermaceae	S/C	*Stephania abyssinica*	Kalala	Nekemte in Oromia region	Roots	Skin cancer	Honey is mixed with the juice prepared from the root of the plant and taken to give relief	[11]
Menispermaceae	S/C	*Stephania abyssinica*	Hidden	Harari region	Roots	NSC	The root of the plant is dried and crushed and pasted and bandaged on the affected area	[21]
Menispermaceae	S/C	*Stephania abyssinica*	Kalala	Wondo genet in SNNP	Leaves	Skin cancer	Fresh leaves of the plant are massaged by hand, and droplets are applied to the affected area	[22]
Menispermaceae	S/C	*Stephani abyssinica*	Yeayethareg	Across the region of Ethiopia	Roots	NSC	The leaf of the plant is boiled, and about one cup is drank for a treatment	[13]
Moraceae	H	*Dorstenia barnimiana*	Work-bemeda	Dekisland in Amhara region and Harari region	Roots, tuber, and aerial parts	Hemorrhoid cancer	Aerial parts of the plant are powdered and made paste with butter and put on the top part of the affected area. To treat the affected area, fresh or dry root is inserted in the opening part	[21, 64]
Moraceae	H	*Dorstenia barnimiana*	Work bemeda	Around Bahir Dar Zuria Woreda in Amhara region	Roots	NSC	The roots of the plant are dried and grounded and mixed with honey and water and drank. Inserting fresh dry root at the affected part	[59]
Moraceae	S	*Dorstenia barnimiana*	Worqbemeda	Bahir Dar Zuria in Amhara region	Root	NSC	Fresh roots of the plant are then crushed and applied	[21, 11]
Myrsinaceae	S	*Myrsine africana*	Quechee	Fiche district in Oromia region	Fruit	NSC	Dried fruit and leaves of plant are powdered and mixed with little water and taken orally	[58]
Phytolaccaceae	S	*Phytolacca dodecandra*	Endod	Bensa and Dawro in SNMP	Leaves and root	NSC	The leaves of the plant are chopped or pounded and applied to the affected part	[11]
Pittosporaceae	S	*Pittosporum abyssinicum*	Lola	Dale district in SNNP	Bark	NSC	The juice made from the bark of the plant is drank for the treatment of cancer	[42]
Plumbaginaceae	S	*Plumbago zeylanica*	Amera	Bahir Dar Zuria in Amhara region	Roots, leaves, and bark	NSC	Powdered together with onion and honey	[65]
Plantaginaceae	H	*Plantago lanceolata*	Yebeglat	Hawassa city in SNNPR	Seed	NSC	The seed of the plant is crushed, powdered, and applied to the affected area	[11]

Table 2: Continued.

Family	Habitat	Plant name	Local name	The region in which the plant can be found	Part of the plant used to treat cancer	Type of cancer	Ethnobotanical preparation	Reference
Plantaginaceae	H	*Plantago lanceolata*	Gorteb	Sidama regional state	Seed	NSC	The dried seeds are powdered, crushed, and put into the cancer wound	[39]
Plumbaginaceae	S	*Plumbago zeylanica*	Amera	Harari region	Roots	Bone cancer	The root is powdered and pasted on the affected area and bandaged	[21]
Plumbaginaceae	S	*Plumbago zeylanica*	Amira	Tigray, Amhara, Oromia region	Roots	NSC	The root of the plant is powder and combined with sulphur and placed on top position or powdered and drank with boiled tea or coffee	[65, 51]
Plumbaginaceae	S	*Plumbago zeylanica*	America	Bahir Dar Zuria in Amhara region	Leaves	NSC	The juice is made from fresh leaves and taken orally	[42]
Podocarpaceae	T	*Podocarpus falcatus*	Bribie	Not specified	Root	NSC	The plant's dry root powder is mixed with water and is taken orally and applied topically to the affected area	[66]
Podocarpaceae	T	*Afrocarpus falcatus*	Zigba	Dale district in Sidamo region	Leaves	NSC	The juice of the leaf is taken for treating cancer	[46]
Podocarpaceae	T	*Afrocarpus falcatus*	Zigba	Dek Island in Amhara region	Root	NSC	Powdered dry root combined with water	[46]
Polygonaceae	H	*Rumex abyssinicus*	Mekumoko	Harari region	Rhizome	Breast cancer	Decocted hot infusion is taken orally	[21]
Polygonaceae	H	*Rumex abyssinicus*	Moke-moko	Seharti Samre in Tigray region	Roots	NSC	The root of the plant is powdered and mixed in a spicy stew and then used	[67]
Polygonaceae	H	*Rumex abyssinicus*	Mem-eqo	Across the region of Ethiopia	Roots	Breast cancer	The root of the plant is powdered and creamed on the affected area of swelling. Decocted hot infusion is taken orally	[21, 11]
Polygonaceae	H	*Rumex nepalensis*	Groucho	Doyo Gena in SNMP	Roots and bark	NSC	The dried roots of the plant are crushed and given with food, or the sap from the fresh bark is crushed and squeezed and then put on the affected area	[43]
Polygonaceae	H	*Rumex nervosus*	Huot/ Embuacho	Seharti Samre district in Tigray region	Leaf	Breast cancer	The leaves of the plant are pounded, and its paste is put on the affected area	[67]
Polygonaceae	H	*Rumex nervosus*	Huot	Seharti Samre in Tigray region	Leaves	NSC	Leaves are crushed and the paste is applied on the affected area	[67]
Punicaceae	T	*Punica granatum*	Roman	Libo Kemke in Amhara region	Fruit	NSC	Crushed the fruit of the plant and eaten	[21]
Ranunculaceae	H	*Ranunculus multifidus*	Etsesiol	Debre Libanos monastery in Oromia region	Roots	NSC	On the affected area, the paste of the root is applied externally	[42]
Ranunculaceae	H	*Delphinium dasycaulon*	Sam-onya	Harari region	Root	NSC	Concoction is taken orally	[21]

Table 2: Continued.

Family	Habitat	Plant name	Local name	The region in which the plant can be found	Part of the plant used to treat cancer	Type of cancer	Ethnobotanical preparation	Reference
Ranunculaceae	H	*Thalictrum rhynchocarpum*	Sire Bizu	Across the region of Ethiopia	Roots	NSC	The root of the plant is mixed with other herbs and put topically	[11]
Ranunculaceae	H	*Ranunculus multifidus*	Etsesiol	Debre Libanos monastery in Oromia region	Leaves	NSC	Externally, the affected area is covered by the powdered leaves of the plant	[59]
Ranunculaceae	H	*Ranunculus multifidus*	Etsesiol	Debre Libanos monastery in Oromia region	Roots	NSC	Powder dressing on the affected area	[59]
Ranunculaceae	S/C	*Clematis hirsuta*	Amazon-hareg	All over Ethiopia	Leaves, stems, and bark	Neck cancer	The plant's bark, leaves, and stems are grounded into a powder that is applied directly on tumour sites	[42]
Ranunculaceae	W/C	*Clematis simensis*	Yeazo-hareg	Libo Kemke/ Amhara region	Leaves	NSC	Crushed and applied	[45]
Ranunculaceae	C	*Clematis virginiana*	Fidy	Bale/Oromia region	Leaves	NSC	Pounding the leaves, making solution, or mixing with butter	[30]
Ranunculaceae	C	*Clematis simensis*	Fireside	Libo Kemkem district/Amhara region	Leaves	Skin cancer	Crush the leaf and add it to the infected area	[42]
Ranunculaceae	C/S	*Clematis hirsuta*	Amazon-hareg	Bale/Oromia region	Leaves, stem, and bark	Neck cancer	The leaves are crushed and used as a bandage on the swelling	[57]
Ranunculaceae	C/S	*Clematis hirsuta*	Amazon-hareg	All over Ethiopia	Leaves, stems, and bark	Neck cancer	The plant's bark, leaves, and stems are grounded into powder that is applied directly on tumour sites	[42]
Ranunculaceae	C	*Clematis simensis*	Yeazo-hareg	Libo Kemke/ Amhara region	Leaves	NSC	Crushed and applied	[45]
Ranunculaceae	C/S	*Clematis hirsuta*	Amazon-hareg	Bale/Oromia region	Leaves, stem, and bark	Neck cancer	The leaves are crushed and used as a bandage on the swelling	[57]
Rhamnaceae	T	*Ziziphus spina-christi*	Geba	All over Ethiopia	The whole parts	NSC	It is used for the treatment of tumour	[42]
Rosaceae	T	*Prunus africana*	Tikurenchet	Bensa in SNNP	Bark and leaves	NSC	Powdered bark of the plant is applied on the skin of the patient to get relief	[68]
Rosaceae	T	*Hagenia abyssinica*	Kosso	Across the regions of Ethiopia	Root	NSC	Honey is mixed to the pounded root of the plant and then creamed on the affected part	[11]

Table 2: Continued.

Family	Habitat	Plant name	Local name	The region in which the plant can be found	Part of the plant used to treat cancer	Type of cancer	Ethnobotanical preparation	Reference
Rosaceae	T	*Prunus africana*	Tikurenchet	Bensa, SNNP	Powdered bark	NSC	Swelling the powdered bark of the plant	[68]
Rubiaceae	C	*Rubia cordifolia*	Enchibir	Gubalafto district in Amhara region	Roots	Lung cancer	The root part of the plant is powdered in water for three days and given orally	[38]
Rubiaceae	C	*Rubia cordifolia*	Enchibir	Across the regions of Ethiopia	Roots	NSC	The root of the plant mixed with other herbs and put topically on the affected area	[11]
Rubiaceae	T	*Pavetta gardeniifolia*	Qadiidaa	Bule Horra in Oromia region	Root	NSC	Pounded and applied	[69]
Rutaceae	T	*Zanthoxylum chalybeum*	Gada	Hawass in SNNR	Leaves	NSC	The leaves of the plant are powdered and drank	[69]
Rutaceae	T	*Fagaropsis angolensis*	Dergi	NA	Fruit	NSC	The juice made from the fruit of the plant is taken orally and applied externally to the affected area	[42]
Rutaceae	S	*Clausena anisata*	Limit	Abay Gorge/ Amhara region	Leaves	NSC	Dry leaves of the plant are powdered and mixed with honey and eaten	[30]
Santalaceae	T/S	*Osyris quadripartita*	Quote	Around Fiche district in Oromia region	Leaves	NSC	The dried leaf of the plant is mixed with dried and grounded fruit of Myrsine africana and combined with water and taken orally	[59]
Sapindaceae	T	*Dodonaea viscosa*	Kitkita	Bahir Dar Zuria in Amhara region	Root	NSC	Honey is mixed with the dried and powdered roots of the plant and drank	[46]
Sapindaceae	T	*Dodonaea angustifolia*	Ketketa	Wide range part in Ethiopia	All parts	Neck cancer	The paste, which is made from whole parts of the plant is put on the affected area	[18]
Sapotaceae	T	*Mimusops kummel*	Safa	Berber district in Oromia region	Root	Lung cancer	The root and fruit of the plant are grounded and dissolved with a small amount of water and taken orally to treat lung cancer	[6]
Sapotaceae	T	*Mimusops kummel*	Galati	Berbere district in Oromia region	Root	Lung cancer	The roots are powdered, and a small amount is ingested with water	[6]
Sapotaceae	T	*Mimusops kummel*	Ishe	Benishangul, Amhara, and Gambela region	Fruit and root	Lung cancer	The root and fruits of the plant are grounded and dissolved with a small amount of water and taken orally	[6]
Sapotaceae	S	*Sideroxylon oxyacanthum*	Bunguude	Dalle district in Sidama region	Leaves	Cancer	The leaf is macerated and given an overall flavour, sometimes with Zanthoxylum chalybeum leaf and honey	[49]
Scrophulariaceae	S	*Verbascumsinaiticum*	Yefereszeng	Dek Island in Amhara region	Roots	Breast cancer	Powder mixed with hyena feces and latex	[22, 42]

Table 2: Continued.

Family	Habitat	Plant name	Local name	The region in which the plant can be found	Part of the plant used to treat cancer	Type of cancer	Ethnobotanical preparation	Reference
Simaroubaceae	T	*Brucea antidysenterica*	Abalo	Jimma in Oromia region	Leaves	NSC	The leaves of the plant are powdered and mixed with young twigs to make pastes and placed on the affected area	[11]
Simaroubaceae	T	*Brucea antidysenterica*	Abalo	Jimma in Oromia region	Steam, bark, and leaves	NSC	Paste is made from leaves and young twigs with water and drank before meals	[42]; [30]
Simaroubaceae	T	*Brucea antidysenterica*	Waginos/ Apollo	Jimma zone and Bale zone in Oromia region	Steam, bark, and leaves	NSC	The decoction is drank, and pastes are made from young twigs and powered leaves with water	[13]
Solanaceae	S	*Discopodium penninervium*	Chechanga	Doyo Gena in SNNPR	Leaves	NSC	Fresh leaves of the plant are crushed and applied on the affected area	[30]
Solanaceae	S	*Solanum nigrum*	Embuayzerech	Across the region of Ethiopia	Leaves, stems, and roots	NSC	The herb is boiled and put in our food daily for about three days	[22]
Solanaceae	S	*Withania somnifera*	Ozawa	NA	Root	NSC	The root is directly chewed orally	[11]
Solanaceae	S	*Lycopersicon esculentum*	Tematim	All over Ethiopia	Fruit	NSC	Without cooking, fresh fruit is washed and ate	[42]
Solanaceae	H	*Solanum americanum*	Tikurawut	NA	Leaves, root, and steam	NSC	Leaves are boiled thoroughly and eaten	[67]
Thymelaeaceae	H	*India involucrata*	Yezinge-rotelba	NA	Roots	NSC	The root of the plant is powdered and made paste with honey	[30]
Verbenaceae	S	*Lantana trifolia*	Hanshi-Bello	Wondo Genet in SNMP	Leaves	NSC	Fresh leaves are powdered and drank after being immersed in cold spring water	[30]
Verbenaceae	S	*Lippia adoensis*	Kessie	Abay gorge in Amhara region	Leaves	NSC	The dried leaves are powdered, soaked in cold water, and drank	[30]
Vitaceae	C	*Rhoicissus tridentate*	Buriguraa	Harari region	Root	NSC	Concoction is taken orally	[21]
Vitaceae	C	*Cyphostemma serpens*	Eirini	Gewan/Afar region	Root	NSC	Dry roots are grounded, then eaten, and added after being pasted with honey	[30]
Zygophyllaceae	H	*Tribulus terrestris*	Camera	Across the regions of Ethiopia	All parts	NSC	The plant is recommended as an anticancer	[70]

NSC: nonspecified cancer; H: herb; S: shrub; T: tree; W: weed; C: climbing plant.

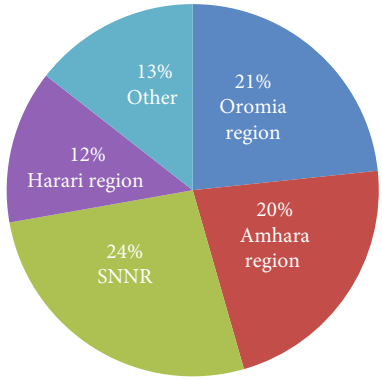


Figure 2: Region-wise distribution of anticancer MPs in Ethiopia.

used to make anticancer remedies were leaves (36.76%), roots (27.2%), bark (12.5%), stem (5.1%), and fruits (7.35%) (Figures 2 and 3).

3.2. Pharmacology Activities to Treat Cancer

3.2.1. Plants Used against Breast Cancer. The most frequent cancer in women worldwide is breast cancer [37]. It is Ethiopia's most common cancer, with high morbidity and mortality rates. The number of new cases increases year to year in the country [71]. According to Memirie et al. [72], of all cancer cases in Ethiopia, 23% accounts for breast cancer. It accounts for 33% of the cancers in women. Breast cancer can be treated scientifically using different MPs. *Aerva javanica*, commonly known as "Tobia," has been confirmed to be used for cancer care. The crude extract from the leaves of *Aerva javanica* has an antiproliferative effect on human breast cancer cell lines (MCF-7) [73]. *Kalanchoe petition*, commonly called "indahul," used to cure breast cancer. The gallic acid isolated from the leave of *Kalanchoe petition* is responsible for its anticancer activity [56]. Extracts of *Sideroxylon oxyacanthum* are reported to be used frequently against breast cancer [49].

In another study, chloroform extract of aerial part of *Clematis simensis* was tested for anticancer activity using MTT assay against three breast cancer cell lines (JIMT-1, MCF-7, and MCF-10A). The IC_{50} (μg/ml) values obtained after treating two breast cancer cell lines (JIMT-1 and MCF-7) and MCF-10A (one normal-like breast epithelial cell line) were as 80 ± 19, 190 ± 70, and 97 ± 9, respectively [74]. *Asparagus africanus*, named "Yeset-kest" in the local Ethiopian language, also treats cancer. The roots of the plant have been reported for treating breast tumors [22]. People of various religious and ethnic groups in Ethiopia use *Aerva javanica* as a traditional medicine to treat multiple diseases, including cancer. A scientifically validated study found that the leaf extracts of *Aerva javanica* showed an antiproliferative effect on human breast cancer cell lines (MCF-7) [42]. Alkaloids isolated from *Catharanthus roseus* showed potent cytotoxicity against the MDA-MB-231 breast cancer cell line, with IC_{50} values ranging from $0.97 \pm 0.07\,\mu$M to $7.93 \pm 0.42\,\mu$M [40]. In another work of Tesfaye and coworkers [75], they checked the cytotoxic activity of *Euphorbia schimperiana*, *Crambe abyssinica*, *Aloe debrana*, *Vachellia nilotica*, *Camellia sinensis*, *Termitomyces schimperi*, *Pentarrhinum insipidum*, *Acmella caulirhiza*, *Leonotis ocymifolia*, *Dorstenia barnimiana*, *Rumex nervosus*, *Clausena anisata*, *Helichrysum mannii*, *Salvia leucantha*, *Vernonia auriculifera*, *Corymbia brachycarpa*, and *Croton macrostachyus* extracts. Out of these, *Euphorbia schimperiana*, *Acokanthera schimperi*, *Kniphofia foliosa*, and *Kalanchoe petition* showed antiproliferative activity against human breast (MCF-7) cancer cell lines.

3.2.2. Plants Used against Lung Cancer. Lung cancer is the leading cause of cancer-related deaths in men and the second leading cause of cancer-related deaths in women after breast cancer in the world [76]. GLOBOCAN 2020 is an online database providing global cancer statistics and estimates of incidence and mortality in 185 countries for 36 types and all cancer sites combined. According to GLOBOCAN data, there were approximately 18.1 million new cancer cases and 9.6 million deaths worldwide in 2018. Of these, 1.76 million died of lung cancer [77]. In the specified year, the number of new lung cancer cases in Ethiopia is 3.1% and it accounts for 4.3% of deaths from the total number of new cancer diseases [20]. Different MPs are used for the prevention and treatment of lung cancer. The seed extracts of *Glinus lotoides* (n-hexane, chloroform, methanol, and water) were tested for anticancer activity on the lung cancer cell line (Calu-3) using MTT assay. The result showed that methanol extract exhibits the highest anticancer activity with an IC_{50} value of $29.7 \pm 1.3\,\mu$g/mL, while water extracts ($IC_{50} = 262.2 \pm 1.2\,\mu$g/mL) exhibit the least anticancer activity [78].

In another study, the anticancer activity of the root of *Aloe pirottae* was tested against stomach cancer (SNU-638), ovarian cancer (A2780), pancreatic cancer (MIA-PaCa-2), and lung cancer (A549) cell lines. The results demonstrated that all extracts exhibited anticancer activity with an IC_{50} value ranging from 6.37 to 29.69 μg/mL [79].

The in vitro cytotoxic activity of essential oils and extracts of *Ocimum basilicum* was tested on a cancerous cell line (MCF-7). The result showed that the cytotoxic activity of essential oil was found to be more effective than that of the extracts [80].

Steroids extracted from *Withania somnifera* leaves were tested for the lung cancer cell line (NCI-H460). The result showed that steroids exhibited suitable anticancer activities with an IC_{50} value of 0.45 μg/mL [81]. The cytotoxic activity T/Corr (%) of the extract (50 μg/mL) on the lung cancer cell line A427 after 96 hours was tested by Tesfaye et al. [75], with a crystal violet cell proliferation test. According to their research result, *Crambe abyssinica*, *Aloe debrana*, and *Vachellia nilotica* showed values of 29.29, 49.65, 26.76, 26.41, and 46.62.

3.2.3. Plants against Blood Cancer (Leukemia). Blood-forming stem cells are the source of all blood cells. Blood cancer is caused by defects in the differentiation of these stem cells, which mainly affect white blood cells. Bone marrow transplantation, chemotherapy, antibodies, cytokines, and tumor vaccinations are choices for improving leukemia patients'

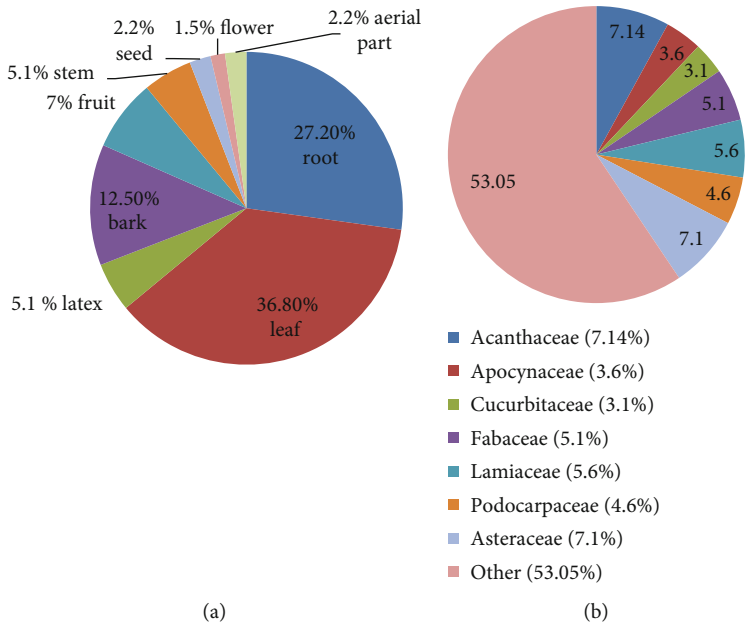


Figure 3: (a) Percent usage of different parts of MPs against cancer; (b) family-wise percentage of anticancer MPs.

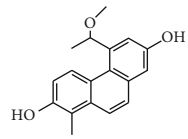
survival rates [37]. Some Ethiopian plants such as *Clerodendrum myricoides*, *Myrsine melanophloeos*, and *Solanecio angulatus* have demonstrated anticancer activity in the case of leukemia [11]. The flower and leaf extracts of *Solanecio angulatus* were tested for anticancer activities against HL-60 human leukemia cell. The flower extract of the plant showed higher anticancer activities against the cell line with an IC_{50} value of 27.39 μg/mL [82]. Essential oils of *Myrtus communis* were reported for the presence of 1,8-cineole, linalool, myrtenyl acetate, and myrtenol which is responsible for its anticancer activity against blood cancer (leukemia) [83]. Methanol and chloroform leaf extracts of *Cynoglossum coeruleum* were tested for anticancer activities against the HL-60 human leukemia cell line. The result indicated that the methanol extracts showed higher anticancer activity (IC_{50} = 183.95 μg/mL) than chloroform extract (312.62 μg/mL). The lowest IC_{50} value was recorded in methanol extract from *Cynoglossum coeruleum* flower with a value of 360.2 μg/mL [82]. In another study, *Jatropha curcas* seed extracts displayed potent inhibition against P388 lymphocytic leukemia (both in vitro and in vivo) [42]. One research report showed that Alkaloids isolated from *Catharanthus roseus* such as vincristine, vinblastine, vindesine, vinorelbine, and vinflunine exhibited cytotoxic activity against human leukemia cells [84]. According to another study, the anticancer activity of crude extracts of *Rumex abyssinicus* roots was observed in prostate, brain, and breast tumor cell lines and leukemia cell culture [22]. Flavonoids, namely, alpinumisoflavone and 4′-methoxylicoflavanone extracted from *Erythrina asuberosa* stem bark, were tested for anticancer activity against HL-60 cells (human leukemia) and the result confirmed their anticancer activity [85].

3.2.4. Plants Used against Skin Cancer. The most common cancer in the world is skin cancer. Melanoma is a type of skin cancer that involves basal and squamous cell carcinomas [37]. According to the WHO data from 2017, skin cancer deaths in Ethiopia accounted for 0.03 percent of all deaths. The age-adjusted death rate is 0.37 per 100,000 people of Ethiopia. The most recent WHO data from 2020 also showed that skin cancer deaths in Ethiopia accounted for 0.21 percent of all deaths, with new cases of 0.31% [86]. Phytochemicals with anti-inflammatory, immune-modulatory, and antioxidant properties have the best chance of acting as a chemopreventive in skin cancers [87]. Scopoletin (7-hydroxy-6-methoxy coumarin) from *Gelsemium sempervirens* has been reported to show anticancer activity against a skin cancer cell line (melanoma A-375) [88]. Plumbagin (a quinonoid constituent) (Figure 4) isolated from the root of *Plumbago zeylanica* was reported as having anticancer activity [89].

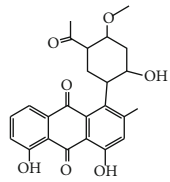
The methanol extract of the leaf of *Plantago lanceolata* was tested for anticancer activity. The result showed anticancer activities on the UACC-62 cell line with an IC_{50} value of 50.58 ± 11.15 μg/mL[90]. Triterpenes found from the root of *Cucumis prophetarum* and gallic acid isolated from leaves of *Kalanchoe petitiana* are also used to cure skin cancer [90]. Bussa and Belayneh [21] reported the ethnomedicinal use of *Vernonia glaberrima* leaves and their phytoconstituents against skin cancer. The crude extract obtained from leaves, stems, and barks of *Clematis hirsute* is used for treating tumor/cancer on the neck [13].

4. Bioactive Compounds Used for Cancer Treatment

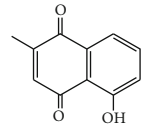
MPs are the source of many secondary metabolites known for their anticancer activity [37]. Phenolic compounds, alkaloids, glycosides, and terpenoids are some examples of such secondary metabolites with anticancer activity [30].



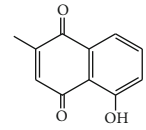
(-) Epigallocathechin5-(1-methoxyethyl)-1-methyl-phenanthren-2,7-diol



Knipholone



Plumbagin



Effususol

Figure 4: Anticancer phenolic compounds.

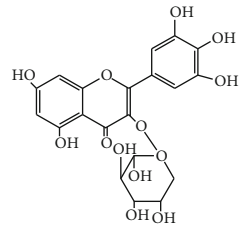
4.1. Phenolic Compound. In plant species, phenolic compounds are formed biologically via flavonoid, phenylpropanoid, and shikimate and possess hydroxide groups in the aromatic ring. These phenolic molecules have been shown for their cytotoxic, antiproliferative, and antioxidant characteristics [91]. Ethiopia has many MPs used to treat cancer; due to the existence of the phenolic molecule, for example, Okoye and coworkers [92] showed the anticancer activity of epigallocatechin extracted from *Maytenus senegalensis*. The bioactive compounds obtained from *Juncus effuses* such as 1-methylpyrene-2,7-diol, dehydrojuncusol, dehydroeffusol, effusol, effususol A, and 5-(1-methoxyethyl)-1-methyl-phenanthrene-2,7-diol (Figure 4) inhibited the proliferation of human cancer cell lines [93, 94]. Naphthoquinone isolated from *Plumbago zeylanica* extracts also treated human pancreatic and lung cancers [95, 96]. In another study, isolated compounds knipholone and knipholoneanthrone from *Kniphofia foliosa* were tested for anticancer activity against leukemic and melanocyte cancer cell lines. The results indicated that knipholoneanthrone has a potential anticancer agent [97].

According to a study on the biological activities and phenolic compounds of ethanolic extracts from *Zingiber officinale* and *Curcuma longa* rhizomes, the plants have anticancer properties in the B164A5 murine melanoma cell line due to the presence of phenolic compounds [98].

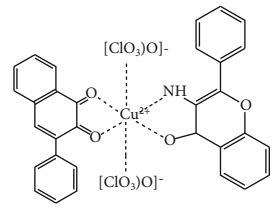
4.2. Flavonoids. Flavonoids are polyphenolic compounds that make up a broad family of secondary metabolites found in plants [85]. Various research showed that flavonoids in different plants had been used for anticancer activities [99]. Multiple studies have shown that increasing the number of flavonoids in one's diet will reduce cancer risk [100]. Quercetin, chalcones, genistein, curcumin, isoflavones, flavanones, and cisplatin are used to treat human oral cancer while daidzein, genistein, quercetin, luteolin, and flavanones are used to treat human breast cancer. Human lung cancer can be treated with flavone and quercetin [101]. Some flavonols like epicatechin, catechin-3-gallate, epigallocatechin flavan-4-ols, flavan-3, 4-diols, flavan-3-ols, catechin, and gallocatechin also are used to treat different cancers such as prostate and rectal cancers. Flavones such as luteolin, chrysin, apigenin, flavonol: rutin, quercetin, myricetin, kaempferol flavanones: naringenin, hesperidin, eriodictyol, flavanonols: taxifolin are used to take care of lung cancer, laryngeal cancer, and breast cancer [102].

In human leukemia cells, flavonoids extracted from *Erythrina suberosa* stem bark such as 4′-methoxylicoflavanone and alpinumisoflavone were found to have cytotoxic effects [85]. Flavonoids extracted from *Cassia Angustifolia*, such as scutellarein, quercimeritrin, and rutin demonstrated considerable anticancer activity against MCF-7, Hep2, and HeLa cell lines, with lower cytotoxicity towards the HCEC cell line [103]. The crude extracts/fractions of *Clerodendrum myricoides*, *Vernonia leopoldi*, *Dovyalis abyssinica*, *Sideroxylon oxyacanthum*, *Clematis longicauda*, *Zanthoxylum chalybeum*, and *Clematis simensis* were tested for anticancer activities and found cytotoxic effects against various breast cancer-derived cell lines [74].

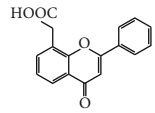
Bioactive compounds such as luteolin, sesquiterpene lactones, coumarins, and phenolic acids isolated from leaves and shoots of *Vernonia amygdalina* have shown cancer chemoprevention [44]. One study observed the anticancer activity in *Cassia angustifolia* extract seed powder against the tested HCEC, Hep2, HeLa, and MCF-7 cell lines. The IC_{50} value of methanol extract against HeLa cells was 5.45 g/L and 4 g/L against MCF-7 cells, lower than the drug taxol 6.07 g/L and tamoxifen 6.4 g/L. This anticancer characteristic is due to bioactive flavonoids such as quercimeritrin, scutellarein, and rutin in the plant's seed [104]. The different ethanolic extracts of *Lagenaria siceraria* were studied for anticancer activity against MCF-7. The result confirmed that it inhibits cancer cells in a concentration-dependent manner with a maximum concentration of 80 μg/mL. This anticancer activity of the extract can be attributed to its flavonoid and polyphenol contents in the extracts [55]. Some of the advanced anticancer flavonoids used to treat cancers are myricetin-3-O alpha-L-rhamnopyranoside, flavone-8-acetic acid, quercetin 3-O-D galactopyranoside, chrysoeriol, nobiletin, silybin, flavopiridol, quercetin-3-O-amino acid-esters, xanthone, indirubin, 5,6 dimethylxanthenone-4-acetic acid, diosmetin, and myricetin-3-O-alpha-L-rhamnopyranoside (Figure 5) [101].



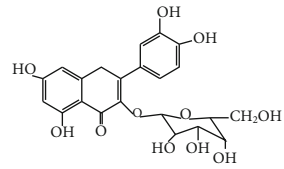
Myricetin-3-O-alpha-L-rhamnopyranoside



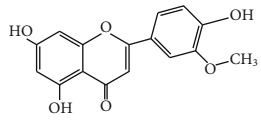
Trans-bis(3-aminoflavone-kappan2N,O bis(perchlorato-kappaO)copper(II)



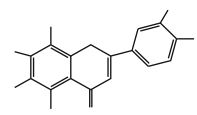
Flavone-8-Acetic Acid (FAA)



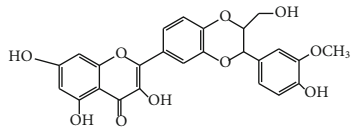
Quercetin 3-O-D-galactopyranoside



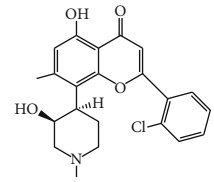
Chrysoeriol



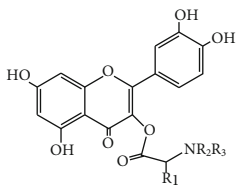
Nobiletin



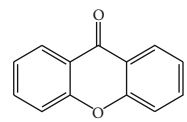
Silybin



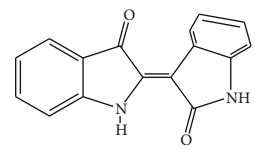
Flavopiridol



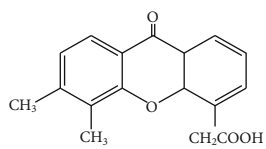
Quercetin -3-O-amino acid-esters



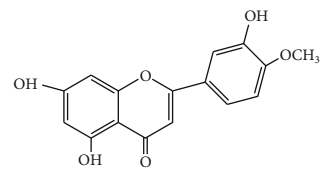
Xanthone



Indirubin



5,6-dimethyxanthenone-4-acetic acid



Diosmetin

Figure 5: Chemical structure of anticancer flavonoids.

4.3. Alkaloids. Alkaloids are essential chemical compounds that can be used to discover new drugs. In vitro and in vivo, some alkaloids derived from natural herbs have antimetastasis and antiproliferative effects on various cancers. Alkaloids including vinblastine and camptothecin have also been used to develop anticancer drugs [104]. The vinca alkaloids, such as vinblastine, vinorelbine, and vincristine, were the first plant-derived anticancer agents to gain approval

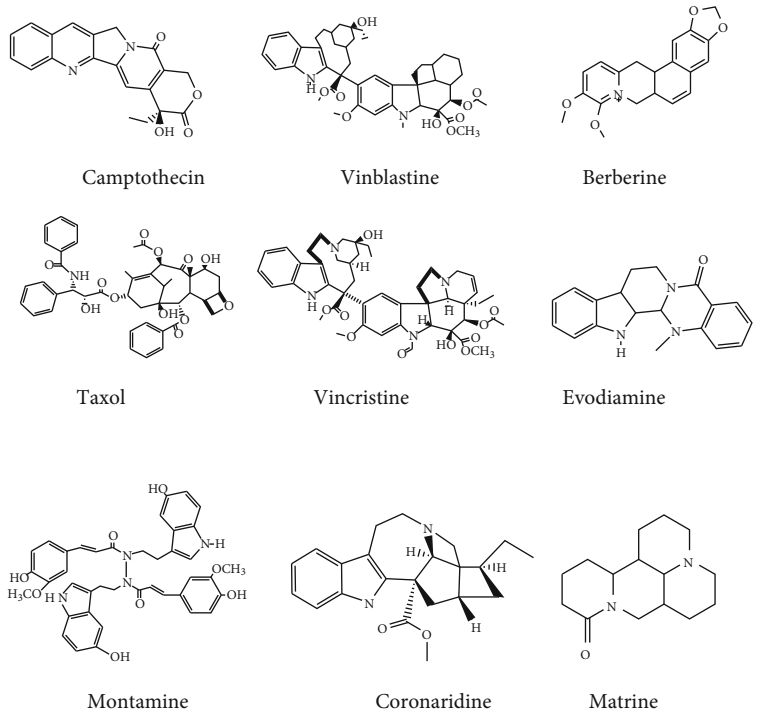


Figure 6: Structure of some anticancer alkaloids.

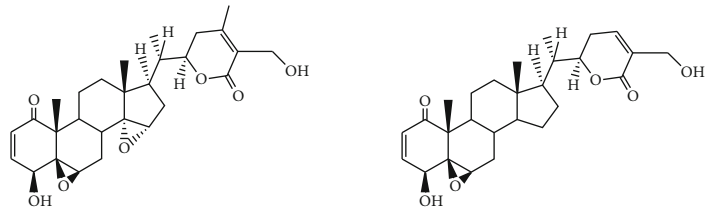


Figure 7: Structure of anticancer steroids.

for clinical use [87]. Some of the alkaloids used having anticancer activities are taxol, vincristine, vinblastine, 9-methoxycamptothecin, berberine, schischkiniin, coronaridine, naucleaorals, monoamine, camptothecin, an indole alkaloid, and protoberberine [105] (Figure 6). In Ethiopia, the alkaloids extracted from the root of *Gloriosa superba* are used to treat breast cancer. When the root is chewed and applied externally to the affected area, it relieves and recovers pain [22, 42]. Phytochemical studies conducted in the Harari region have shown that the alkaloids and glycosides in the roots of *Hydnora abyssinica* are vital for cancer treatment [38]. The chloroform extract of *Clematis simensis*, rich in alkaloid bioactive compounds, showed cytotoxicity against three breast cancer cell lines. Two breast cancer cell lines JIMT-1 and MCF-7 showed IC_{50} values of 80 μg/mL and 190 μg/mL, respectively. One of the normal-like breast epithelial cell lines (MCF-10A) has 97 μg/mL [74]. The alkaloids, which are also present in the flower of *Solanecio angulatus*, showed in vitro cytotoxicity properties with an IC_{50} value of 133.72 μg/mL in the tested cell line (HL-60) [30]. Solasonine and solamargine alkaloid (Figure 6) molecules, which were isolated from *Solanum nigrum*, exhibited anticancer activities on the human gastric cancer cell line (MGC-803) with IC_{50} values of 5.2 μg/mL and 8.77 μg/mL, respectively [40].

4.4. Steroids. A group of natural or synthetic organic compounds with a molecular structure of 17 carbon atoms grouped in four rings is known as steroids. In genetics, chemistry, and medicine, steroid hormones play a significant role. Hundreds of steroids have been discovered in fungi, animals, and plants [106]. Medicinal plant steroids are well-known secondary metabolites to have anticancer activity [107]. Bioactive compounds of steroids which were isolated from *Withania somnifera* such as 5,6,14,15 diepoxy-4,27-dihydroxy-1-oxowitha-2,24-dienolide and withaferin-A (Figure 7) showed anticancer activity to the human lung cancer cell line (NCI-H460) with 0.45 μg/mL and 8.3 μg/mL IC_{50} values, respectively, [81]. In addition, cytotoxic activities were shown in extracts of *Bersama abyssinica*.

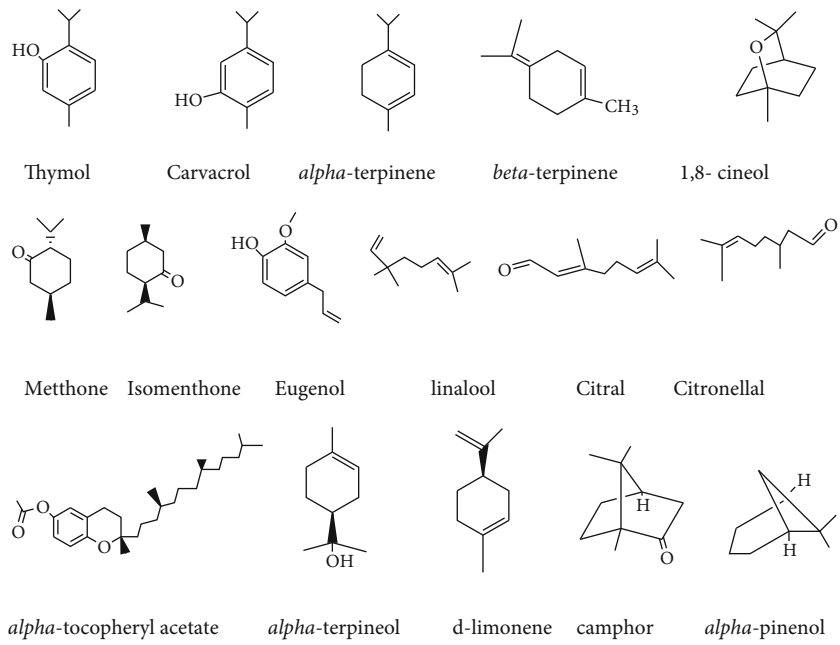


Figure 8: Anticancer components from different essential oil.

Hellebrigenin 3,5 diacetate, hellebrigenin 3-acetate, bersenogenin, 3-epiberscillogenin, and berscillogenin demonstrated cytotoxic activities in the plant extract [108, 109]. Physalin B, D, F steroids which are found in *Physalis angulate* showed anticancer activities on different cancer cell lines such as KB, A549, HCT8, and PC3 with the lowest EC_{50} (μg/mL) value of 0.9 (for KB), 1.3 (for A549), 1.0 (for HCT8), and 0.9 (for PC3), respectively, for physalin F, physalin D, and physalin B [110].

4.5. Essential Oil. Essential oils (EOs) are well-known anticancer bioactive compounds obtained from medicinal and aromatic plants. Essential oils are highly volatile, aromatic yields obtained from plants. Due to their volatility, they can easily be extracted by steam distillation from different natural sources [111]. They may be a generic word for the liquid and highly volatile plant constituents with a distinct odor [111]. EOs having anticancer properties are listed in Figure 8. They are present in plants as secondary metabolites in their flowers, leaves, fruits, buds, seeds, rhizomes, barks, and roots [112, 113]. The essential oils such as limonene and perillyl alcohol, which is extracted from *Citrus sinensis*, are used for anticancer activities [114], and terpinene-4-ol, α-thujone, β-citronellal, α-pinene, γ-eudesmol, δ-cadinene, and methyl cinnamate from the Lamiaceae family are used for anticancer activities [115] as illustrated in Figure 8. The presence of bioactive compounds such as citronellyl acetate, pulegol, and citronellol in essential oils from *Pulicaria inuloides* was used for anticancer activity against liver, breast, and colorectal/colon cancers [116]. The essential oils derived from the flower of *Achillea ligustica*, leaf and the seed of *Coriandrum sativum*, leaf of *Melaleuca alternifolia*, the seed of *Nigella sativa*, and aerial parts of *Pelargonium graveolens* are used to treat different cancer diseases [117]. Some bioactive compounds such as linalool, 1,8-cineole, myrtenyl acetate, and myrtenol in *Myrtus communis* essential oil have anticancer properties in the case of blood cancer (leukemia) [83].

4.6. Other Bioactive Compounds. Various studies have shown that bioactive compounds such as fucoxanthin can be used to prevent breast cancer and triterpenes, anthocyanins, and saponins can be used to treat lung cancer. Blood cancer can be prevented using epigallocatechin gallate and rosavin [118]. Various compounds have been isolated from *Bersama abyssinica* to determine the plant's anticancer or antitumor function. Lignin and hallebergenin 3-acetate are two of these compounds that have been shown to inhibit tumor growth [22]. Garcinol, limonoids, crocin, and genistein are used to prevent pancreatic cancer [118]. The root of *India involucrate*, also known as "Yezngerotelba" in Amharic, can treat cancer, including diterpenes, and gnidicin, mezerein, gnidilatidin, gnidiglucin, and gniditrin are used to prevent cancer isolation biologically active compounds [22]. Boswellia acids in boswellia species give a defense mechanism to have anticancer activities [119]. The presence of gallic acid isolated from the leaves of *Kalanchoe petition*, which is commonly called "indahula," is also essential to cure breast cancer [56]. It has been documented that the roots of *Asparagus africanus* are used to treat tumors [22]. Three lignans isolated from *Carissa spinarum*, namely, nortrachelogenin, carol, and carissanol, were found to be cytotoxic to WI38, MCF7, and A549 cell lines. Compared with carissanol and nortrachelogenin, carinol shows higher cytotoxic activity against these three cell lines, with an IC_{50} value of 1 μg/mL [40].

5. Conclusion and Future Perspective

Several plant species are already being utilized to treat or prevent cancer. Multiple studies have identified plant species with

anticancer characteristics, emphasizing herbal medicine in developing nations. In Ethiopia, many MPs can treat various types of cancer, such as breast cancer, lung cancer, blood cancer, and skin cancer and tumors. The ethnobotanical application of MPs for cancer treatment confirmed that plant leaves are the most valuable for preparing anticancer drugs (36.76%), followed by roots (27.2%), bark (12.5%), and flowers (1.5%). According to the analyzed data, the Euphorbiaceae family has the highest percentage (10.71%) of plant families utilized to treat cancer. The Asteraceae and Lamiaceae families have the second (7.1%) and third (6.1%) values, respectively. Regarding their habit, shrubs account for (39.5%) followed by herbs (33.8%), trees (17.9%), and climber or weed (8.8%).

Although numerous MPs have been utilized ethnobotanically to treat cancer, only a few MPs have been formally examined for anticancer activity. A few secondary metabolites and pure isolated compounds have been tested against cancer cell lines in vitro. Therefore, it is imperative to conduct detailed phytochemical research to isolate new anticancer drugs. Since the traditional knowledge for anticancer medicines provides basic information for further scientific research on the synthesis of anticancer drugs, it is necessary to conduct comprehensive ethnomedicinal research. The anticancer mechanism of these medicinal plant extracts is still unclear. Therefore, more in-depth scientific research is needed, which is the homework for researchers to conduct further studies.

Abbreviations

AIF: Alpinumisoflavone
FAA: Flavone-8-acetic acid
MLF: 4′-Methoxylicoflavanone
MP: Medicinal plant
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium
SRB assay: Sulforhodamine B
EOs: Essential oil
NSC: Nonspecified cancer
SNNP: Southern Nations, Nationalities, and peoples
TMs: Traditional medicines
WHO: World Health Organization.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

LA and MG drafted the review. AB prepared the different tables and figures required for the manuscript. RKB provided guidance during the development of the idea and wrote and revised the manuscript. The authors read and approved the final manuscript.

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Research Article

Propylene Glycol Caprylate-Based Nanoemulsion Formulation of Plumbagin: Development and Characterization of Anticancer Activity

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Plumbagin, a bioactive naphthoquinone, has demonstrated potent antitumor potential. However, plumbagin is a sparingly water-soluble compound; therefore, clinical translation requires and will be facilitated by the development of a new pharmaceutical formulation. We have generated an oil-in-water nanoemulsion formulation of plumbagin using a low-energy spontaneous emulsification process with propylene glycol caprylate (Capryol 90) as an oil phase and Labrasol/Kolliphor RH40 as surfactant and cosurfactant excipients. Formulation studies using Capryol 90/Labrasol/Kolliphor RH40 components, based on pseudoternary diagram and analysis of particle size distribution and polydispersity determined by dynamic light scattering (DLS), identified an optimized composition of excipients for nanoparticle formulation. The nanoemulsion loaded with plumbagin as an active pharmaceutical ingredient had an average hydrodynamic diameter of 30.9 nm with narrow polydispersity. The nanoemulsion exhibited long-term stability, as well as good retention of particle size in simulated physiological environments. Furthermore, plumbagin-loaded nanoemulsion showed an augmented cytotoxicity against prostate cancer cells PTEN-P2 in comparison to free drug. In conclusion, we generated a formulation of plumbagin with high loading drug capacity, robust stability, and scalable production. Novel Capryol 90-based nanoemulsion formulation of plumbagin demonstrated antiproliferative activity against prostate cancer cells, warranting thus further pharmaceutical development.

1. Introduction

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone, Figure 1) is a naturally occurring 1,4-naphthoquinone found in Plumbaginaceae and other plant families [1]. Plumbagin has a variety of pharmacological properties that include potent antitumor, antiatherosclerotic, anti-inflammatory, antibacterial, antifungal, and neuroprotective activities [1–3]. It has demonstrated broad-spectrum anticancer efficacy on diverse types of cancer, including glioma, hepatocellular carcinoma, melanoma, and promyelocytic leukemia, as well as breast, esophageal, lung, ovarian, and prostate cancer [4].

Numerous investigations have indicated that the anticancer activity of plumbagin is mediated through effects via several signal transduction pathways or targets, including AMPK, CDK1/CDC2, cyclin B1, cyclin D1, FOXM1, NF-κB, p53, p21 Waf1/Cip1, p27 Kip1, PI-5, Nrf2/ARE, PI3K/AKT/mTOR, Ras, Sirt1, STAT3/PLK1/AKT, and Wnt [3]. Furthermore, plumbagin is an efficient generator of reactive oxygen species (ROS), inducing oxidative stress in tumor cells [5, 6]. These activities contribute to the anticancer effects of plumbagin, leading to cell cycle arrest and apoptotic death of tumor cells or inhibition of metastatic activity [2].

Thus, plumbagin is a promising drug that has drawn a significant interest in anticancer research, leading to the preclinical development of a new therapeutic drug, currently in clinical trial for the treatment of prostate cancer. Initial studies of the effect of plumbagin in prostate cancer came from Dr. Verma's group, which showed that plumbagin inhibited tumor growth rate or significantly delayed disease

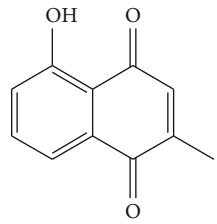

Figure 1: Chemical structure of plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone or 5-hydroxy-2-methyl-1,4-naphthalenedione, $C_{11}H_8O_3$).

progression in various models of prostate cancer [7–9]. A key finding from our group showed that plumbagin significantly improved the efficacy of androgen deprivation therapy (ADT) in hormone-responsive models of prostate cancer, leading to tumor regression and extended survival when used in combination with ADT [10, 11]. These preclinical studies established the framework for the first-in-human clinical trial of the combination of plumbagin and ADT in prostate cancer patients (NCT03137758).

Clinical studies of plumbagin, however, have been impeded by its poor solubility in water. Indeed, the rate and extent of a drug absorption and bioavailability following per-oral administration are greatly affected by its solubility [12], and successful clinical deployment of plumbagin will require a formulation that considerably increases its solubility. Therefore, we have developed and characterized a new nanoemulsion-based drug delivery system of plumbagin. Nanoemulsions are biphasic dispersions of oil in water stabilized by amphiphilic surfactant/cosurfactant interfacial film exhibiting optical isotropy and kinetic stability [13]. Particle size of nanoemulsions is typically within the submicron range, 5-200 nm [13]. Importantly, nanoemulsion formulations have critical advantages for oral drug delivery, including high encapsulation capacity and high surface-to-volume ratio, as well as favorable physicochemical properties facilitating their stability. They have been shown to improve drug pharmacological profiles, reducing toxicity, and potentially improving the stability and pharmacokinetics of encapsulated drugs [14, 15].

This study describes the formulation of nanoemulsions using biocompatible components, with propylene glycol caprylate (Capryol 90) as an oil phase and Labrasol/Kolliphor RH40 as surfactant/cosurfactant excipients. High loading capacity for plumbagin, long-term stability, retention of particle size in simulated physiological environments, and increased sensitivity to the antiproliferative activity of plumbagin in prostate cancer cells indicate that Capryol 90-based nanoemulsion formulations of plumbagin are suitable for further development.

2. Materials and Methods

2.1. Materials. Capryol 90 (propylene glycol caprylate), Labrasol (PEG-8 caprylic/capric glycerides), Labrafil M 1944 CS (mono-, di-, and triglycerides and PEG-6 mono- and diesters of oleic acid), Labrafac lipophile WL 1349 (triglycerides of caprylic and capric acids), and Peceol (glyceryl monooleate) were purchased from Gattefosse (Saint-Priest, France). Capmul MCM (mono-diglyceride of caprylic and capric acid) and Capmul PG-12 (propylene glycol monolaurate) were supplied by Abitec Corporation (Columbus, OH). Glyceryl trioleate, Kolliphor RH40 (polyoxyl 40 hydrogenated castor oil), Span 80 (sorbitan monooleate), Span 85 (sorbitan trioleate), plumbagin, and other chemicals were supplied by Sigma (St. Louis, MO).

2.2. Determination of Plumbagin Solubility in Pharmaceutical Excipients. The solubility of plumbagin in a series of lipid-based pharmaceutical excipients was determined by UV-VIS spectrophotometry as described previously [16, 17]. Briefly, supersaturated dispersions of plumbagin in excipients were incubated at 25°C for 72 h on a rotary shaker. Samples were centrifuged at 10,000 × g for 10 min, and for each sample, the supernatant was filtered through a syringe filter (0.45 μm). Aliquots of filtrates were dispersed in 200–3000-fold volume excess of methanol. The concentration of plumbagin was determined using absorption spectrophotometry (DU-640 spectrophotometer, Beckman Coulter, Fullerton, CA) with molar absorption coefficient for plumbagin determined in methanol, ε_λ (410 nm) = 3,800 $dm^3 \cdot mol^{-1} \cdot cm^{-1}$).

2.3. Construction of Pseudoternary Phase Diagram. A pseudoternary phase diagram was generated as described previously [18] by water titration of mixtures of Capryol 90 and increasing amounts of the Labrasol/Kolliphor R-H40 surfactant/cosurfactant blend at 25°C, in concordance with the common requirements for preparation, storage, and application of nanoemulsions. To identify the nanoemulsion domain, the mixtures were visually inspected for clarity to delineate boundaries of phases and further characterized by dynamic light scattering as described below. Particle size distribution was measured directly without further dilution.

2.4. Particle Size Analysis. Particle size distribution and polydispersity of emulsified formulations were determined by dynamic light scattering (DLS) using noninvasive back scatter (NIBS) detection at a 173° angle and at 25°C on Zetasizer Nano-ZS (Malvern Instruments) equipped with 4 mW He-Ne, 633 nm laser. *Z*-average size (nm) or harmonic intensity averaged particle diameter was calculated by cumulant analysis of autocorrelation function generated from DLS measurement as defined by ISO 13321 and ISO 22412.

2.5. Preparation of Plumbagin-Loaded Nanoemulsions. Plumbagin-loaded nanoemulsions were prepared by low-energy spontaneous emulsification [19]. Briefly, plumbagin was dissolved in Capryol 90 in the range 0.4–4.7% (*w*/*w*) and mixed for 30 min at 25°C. The solution of plumbagin in Capryol 90 was then mixed with a blend of excipients Labrasol/Kolliphor RH 40 (1 : 1 *w*/*w*) at surfactant-cosurfactant to Capryol 90 ratio of 1.35. This organic phase with plumbagin and excipient mixture was mixed on a magnetic stirrer for 10 min at 25°C. Nanoemulsions were then produced by dispersing of the organic phase into deionized water in one step to the final concentration of water, 40.5% (*w*/*w*). Dispersions were vortexed for 10 sec at 25°C.

2.6. Physical Stability of Nanoemulsions. Control and plumbagin-loaded nanoemulsion formulations were analyzed for changes in particle size distribution over time. To study stability in simulated physiologic environments, the formulations were dispersed in 0.1 M HCl and 0.01 M sodium phosphate buffer pH 6.8 and pH 7.5, with 100-fold dilution. The particle size distribution was measured at designated time-points by DLS using Zetasizer Nano-ZS (Malvern Instruments) as described above.

2.7. Cell Culture. PTEN-P2 murine prostate cancer cell line was previously characterized and kindly supplied by Dr. Wu Laboratory [20]. The cells were grown in phenol red-free RPMI-1640 medium (Sigma) supplemented with 10% (*v*/*v*) heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin 100 μg/ml streptomycin, insulin-selenium-transferrin (5 μg/ml insulin), and 10^{-8} mol/l dihydrotestosterone. Cultures were passaged by dissociation with trypsin (0.05%) and maintained at 37°C in a humidified atmosphere with 5% CO_2.

2.8. In Vitro Cytotoxicity Assay. The cytotoxicity of plumbagin formulations was examined using the MTS tetrazolium compound-based method. PTEN-P2 cells were seeded at a density of 8×10^3 cells/well in 96-well plates in replicates ($n = 4$). Then, 24 h later, the medium was changed for medium containing free plumbagin or plumbagin formulations corresponding to the concentration range of 0-10 μmol/l plumbagin. Free plumbagin control was prepared by dissolving plumbagin in dimethylsulfoxide (DMSO). DMSO was kept at constant concentration (0.1% *v*/*v*) within the tested concentration range of plumbagin. Control formulation without plumbagin was analyzed at the same concentration range. The antiproliferative effect was determined after 24 h incubation using the CellTiter 96 AQueous one solution cell proliferation assay kit according to the manufacturer's instructions (Promega, Madison, WI) by measuring the conversion of (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt) to formazan product. Half-maximal inhibitory concentration (IC_{50}) values were interpolated from cytotoxicity curves as the concentration that induced 50% inhibition of the cell growth.

3. Results

3.1. Selection of Excipients and Formulation Development. In order to select the optimal constituents of a nanoemulsion formulation for plumbagin, we first measured the solubility of plumbagin in various lipid-based excipients. As shown in Table 1, excipients based on medium chain glycerides such as Labrasol, Labrafac, and Capmul MCM, or on propylene glycol esters such as Capryol 90, have a remarkably high capacity to solubilize plumbagin. Indeed, plumbagin concentrations in these excipients are higher than 80 mg/ml, reaching 125mg/ml in Labrasol, compared to 50-60 mg/ml range in glyceryl trioleate, Peceol, Span 80, or Span 85. This is much higher than the solubility achieved in organic solvents, which ranges from 22 mg/ml in DMSO to 48 mg/ml in ethyl acetate [10, 11]. It is also higher than the solubility achieved in plant-derived oils, which reaches a maximum of 57.1 mg/ml in sesame oil and is the excipient used in proof-of-efficacy animal studies and first-in-human clinical trial [10, 11].

Table 1: Solubility of plumbagin in lipid-based pharmaceutical excipients at 25°C.

Excipient	Plumbagin mg/ml	±SD
Capryol 90 (propylene glycol monocaprylate (type II) NF)	94.6	0.4
Labrasol (caprylocaproyl macrogol-8 glycerides)	125.0	1.2
Labrafil M 1944 CS (oleoyl macrogol-6 glycerides)	82.1	0.5
Labrafac lipophile WL 1349 (caprylic/capric triglyceride IIG)	89.4	0.1
Capmul MCM EP/NF (glycerol monocaprylocaprate, type I)	92.0	0.6
Capmul PG-12 EP/NF (propylene glycol monolaurate)	83.7	0.1
Glyceryl trioleate	58.6	0.3
Peceol (glyceryl monooleate, type 40)	52.0	0.1
Span 80 (Sorbitan monooleate)	53.5	0.5
Span 85 (Sorbitan trioleate)	57.9	0.1

Based on preformulation studies of the miscibility of various systems of oils and surfactants, their dispersibility in water, and on results of plumbagin solubility shown in Table 1, Capryol 90 (propylene glycol monocaprylate) was selected as an oil phase and Labrasol/Kolliphor RH 40 as a nonionic surfactant/cosurfactant mixture for the development of a new nanoemulsion formulation.

To identify the monophasic, optically isotropic region of the nanoemulsion and to determine the optimal concentration of excipients, a series of mixtures of containing various ratios of Capryol 90 with surfactant Labrasol/cosurfactant Kolliphor RH 40 (1 : 1, *w*/*w*) were prepared. The pseudoternary phase diagram, shown in Figure 2(a), was constructed using progressive water titration as described in Materials and Methods. The established pseudoternary phase diagram was then used to delineate the nanoemulsion domain and the boundary of phases. Samples falling within the nanoemulsion domain appeared translucent whereas mixtures with higher ratios of the Capryol 90 oil phase led to the formation of opalescent coarse dispersions (Figure 2(b)).

Aqueous dispersions of samples with different surfactant/cosurfactant-to-oil (S-CoS/O) ratios were further characterized by photon correlation microscopy (PCS) using Zetasizer Nano-ZS (Malvern). PCS, dynamic light scattering based at 173° backscattering angle, was used to estimate the particle size distribution and the polydispersity of each formulation (Figure 3). *Z*-average, an intensity-based harmonic mean determined by method of cumulants, clearly showed dependency on the S-CoS/O (Figure 4). Figures 3 and 4 show that the hydrodynamic diameter fell into the range of nanoemulsions at S – CoS/O > 1.2 with a low polydispersity index (PDI). Analysis of particle size distribution over time

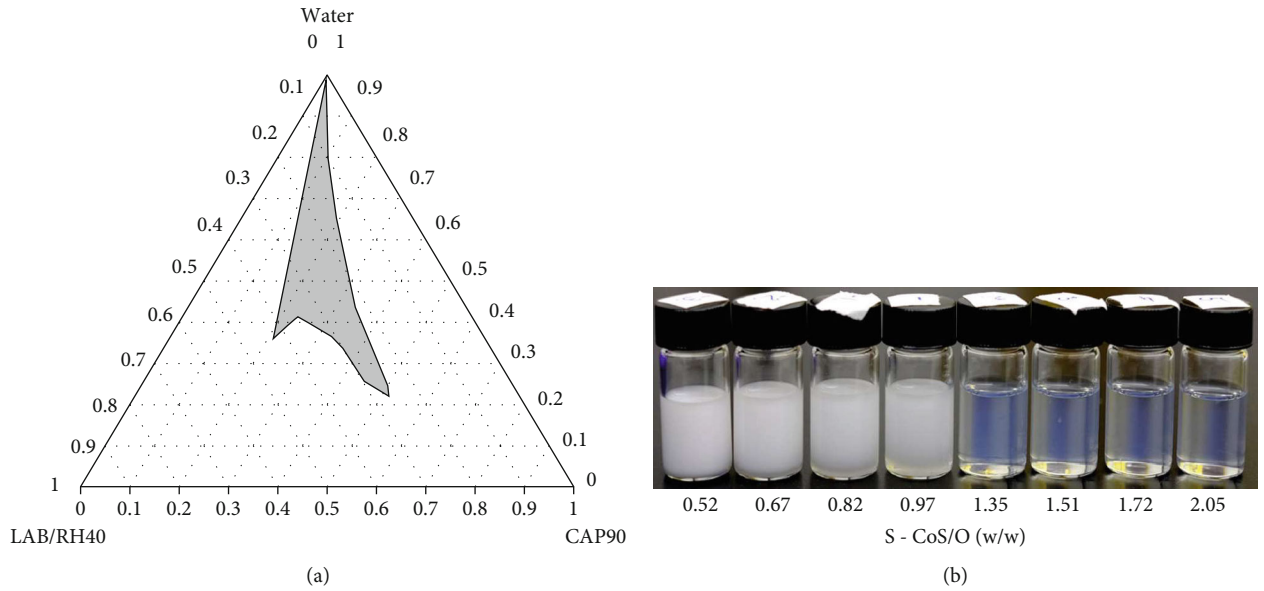

Figure 2: Pseudoternary phase diagram (a) of Capryol 90, Labrasol/Kolliphor RH 40 (1 : 1), and water at 25°C. The grey area indicates o/w nanoemulsion domain. (b) Shows the samples with different w/w ratio of surfactant–cosurfactant to oil (S–CoS/O) ranging from opalescent coarse emulsions to optically translucent nanoemulsions.

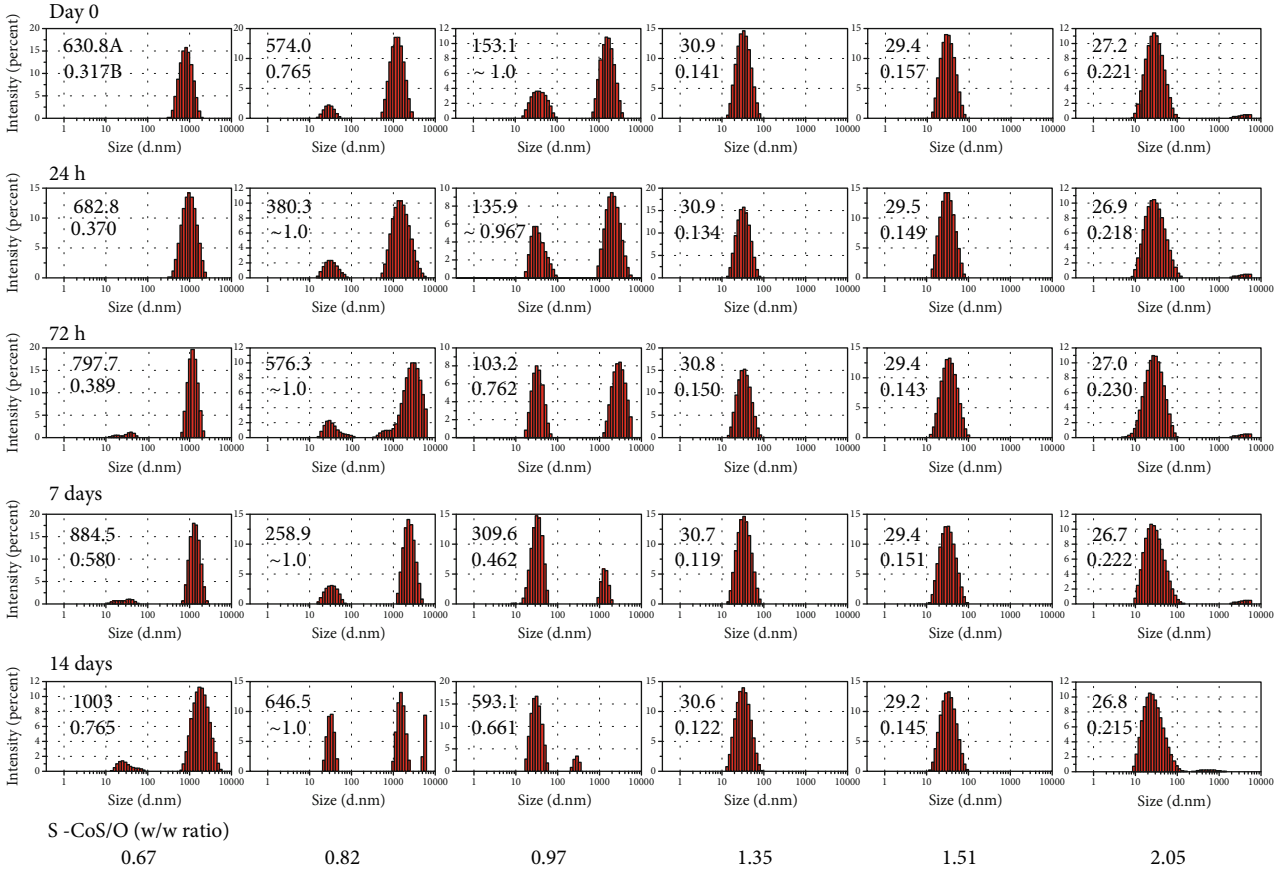

Figure 3: Time-dependent intensity-based particle size distribution profiles (hydrodynamic radius, *Z*-average, nm) and polydispersity index (PDI) of Capryol 90-Labrasol/Kolliphor RH 40 (1 : 1) at different surfactant-cosurfactant to oil (S-CoS/O) (*w*/*w*) ratios.

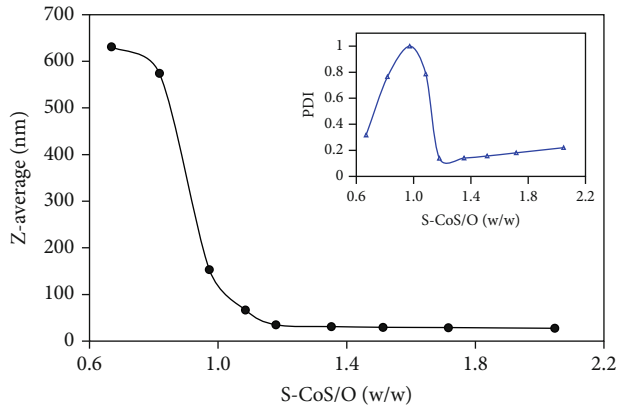

FIGURE 4: Hydrodynamic radius (*Z*-average, nm) and PDI of Capryol 90-Labrasol/Kolliphor RH40(1 : 1) dispersions as a function of surfactant-cosurfactant to oil (S-CoS/O) (*w*/*w*) ratio.

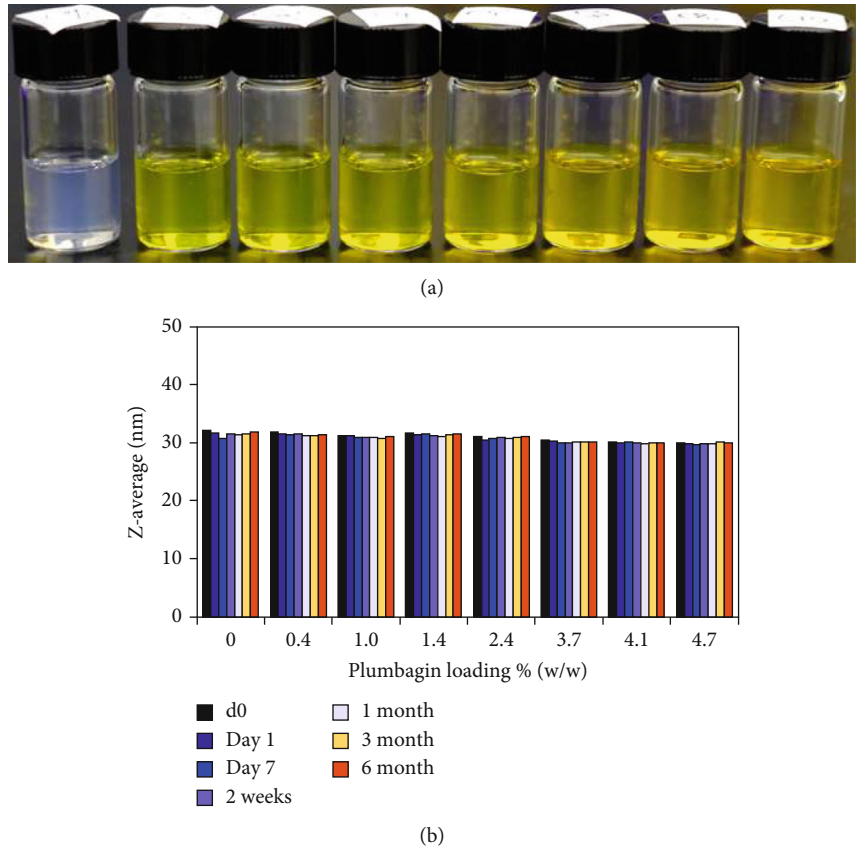

FIGURE 5: Stability of size distribution (hydrodynamic radius over time) of plumbagin-loaded Capryol 90-Labrasol/Kolliphor RH40 (1 : 1) nanoemulsions.

for samples with S – CoS/O < 1 showed a very broad size distribution profile and high polydispersity and instability, while formulations in which the S-CoS/O ratio was within the range of 1.35–1.7 showed a narrow PDI and stability of size distribution over time.

3.2. Stability of Plumbagin-Loaded Nanoemulsion. The nanoemulsion formulation consisting of an S-CoS/O ratio of 1.35 was selected for further development. Nanoemulsions loaded with increasing amounts of plumbagin (0.4–4.7% *w*/*w*) as well as an "empty" formulation without plumbagin displayed similar particle size distributions immediately after production (Figure 5). Both the control and the plumbagin-loaded nanoemulsions showed retention of size distribution during six months of storage at 25°C (Figure 5(b)), indicating good stability over extended period of time.

Finally, the stability of the nanoemulsions was measured in simulated physiological environments. Results presented in Table 2 showed good retention of particle size for both the

Table 2: Stability of nanoemulsions in simulated physiological environments. Changes in particle hydrodynamic radius and PDI of control (NE-C, empty) and plumbagin-loaded (NE-PL, 4.7% *w*/*w*) nanoemulsions were monitored by DLS after dispersion in indicated media and incubation for 24 h at 37°C. PDI values are shown in brackets.

	Z-average hydrodynamic radius (nm) and PDI in different media			
	Water	HCl 0.1 M	0.01 M NaH_2PO_4/Na_2HPO_4 pH 6.8	pH 7.5
NE-C (control)	32.2 (0.180)	30.6 (0.098)	32.4 (0.173)	33.8 (0.165)
NE-PL (loaded)	30.0 (0.162)	30.1 (0.135)	30.0 (0.142)	31.74 (0.135)

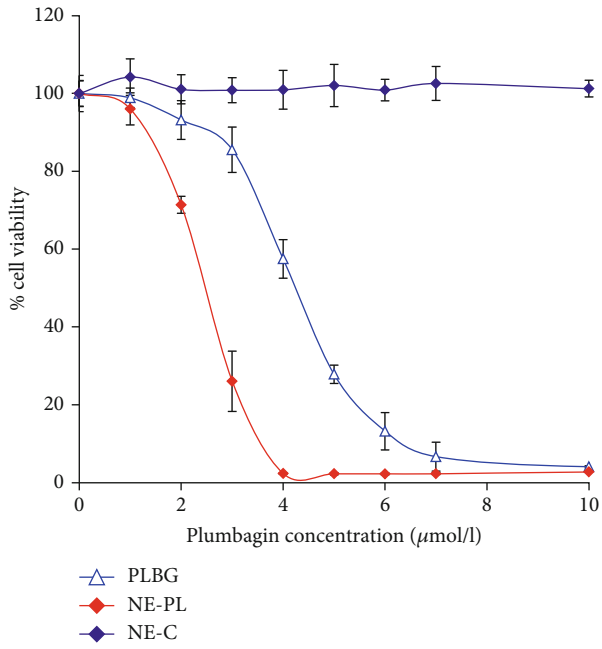

Figure 6: *In vitro* antiproliferative activity of plumbagin-loaded nanoemulsion. Effect of free plumbagin (PLBG), plumbagin-loaded nanoemulsion (NE-PL, 4.7% *w*/*w*), and control empty nanoemulsions (NE-C) on cell viability of PTEN-P2 cells was determined in a dose-dependent manner. For control drug-free nanoemulsion, NE-C, the cells were exposed to the same concentration of nanoemulsion without plumbagin. PTEN-P2 cells were incubated with increasing dilutions of formulations for 24 h, and then, % cell viability was determined using cell proliferation assay (MTS).

control (empty) and the plumbagin-loaded nanoemulsions when dispersed in media that simulate the physiological environment of stomach acid and intestinal fluids.

3.3. In Vitro Antiproliferative Activity of Plumbagin-Loaded Nanoemulsion. The cytotoxicity of the plumbagin-loaded nanoemulsion was evaluated by exposing prostate cancer cells PTEN-P2 to increasing dilutions of the formulation with and without plumbagin for 24 h. Figure 6 demonstrates the dose-dependent cytotoxicity of plumbagin-loaded nanoemulsion compared to the cytotoxicity of free plumbagin. The plumbagin-loaded nanoemulsion formulation displayed higher inhibitory effect on proliferation of PTEN-P2 cells compared to free plumbagin, with IC_{50} 2.5 vs. 4.3 $umol \cdot l^{-1}$, respectively. These findings correlate with an assessment of cell gross morphology, showing that the plumbagin-loaded nanoemulsion formulation induced substantial cell detachment, shrinkage, and cellular damage compared to free plumbagin (Figure 7). Control, drug-free nanoemulsion did not demonstrate notable cytotoxicity in the analyzed range (Figures 6 and 7).

4. Discussion

The majority of therapeutic chemical entities in drug development have poor aqueous solubility [21, 22], which is a significant challenge limiting potential clinical translation. Recent years have seen a mounting effort to bring new drug solubilization and delivery technologies at an earlier stage of the preclinical development process in order to facilitate successful progression into the clinic [23].

Several drug delivery systems have been explored as carriers to formulate plumbagin and potentially improve its anticancer activity, including niosomes, serum albumin, silica nanoparticles, chitosan, poly (lactic-co-glycolic) acid (PLGA), micelle-based systems, gold nanoparticles, and liposomes [24–30]. While these studies showed promising results, production of most of these formulations is costly and/or requires the use of organic solvents or components that are not biocompatible, which limits their translational application. In order to develop a simple, cost-effective, and scalable formulation, we have focused on a nanoemulsion formulation designed using biocompatible pharmaceutical excipients. Furthermore, nanoemulsion formulations have demonstrated excellent absorption and bioavailability, enhanced penetration of biological membranes, and lower inter- and intraindividual variability in drug pharmacokinetics compared to naked drugs [14, 15]. Last but not least, nanoemulsions are compatible with per oral administration, which is highly desirable as the per oral route results in better patient compliance compared to other routes of administration. In addition, per oral administration does not require hospital stay and therefore is more accessible to patients, especially low-income patients with restricted access to health care, as well as patients living in rural or undeveloped areas.

Our preformulation studies identified medium-chain glyceride-based excipients with a high solubilization capacity for plumbagin. A stable formulation of plumbagin was designed using Capryol 90 as an oil phase and Labrasol/Kolliphor RH40 (1 : 1) as surfactant and cosurfactant. Capryol 90 has been used to develop nanoemulsion-based drug delivery systems with particle size distribution comparable to our formulation [31–33]. The plumbagin-loaded nanoemulsion (30.9 nm *Z*-average, Capryol 90 to Labrasol/Kolliphor 40; 1.35 *w*/*w*) demonstrated good retention of size distribution

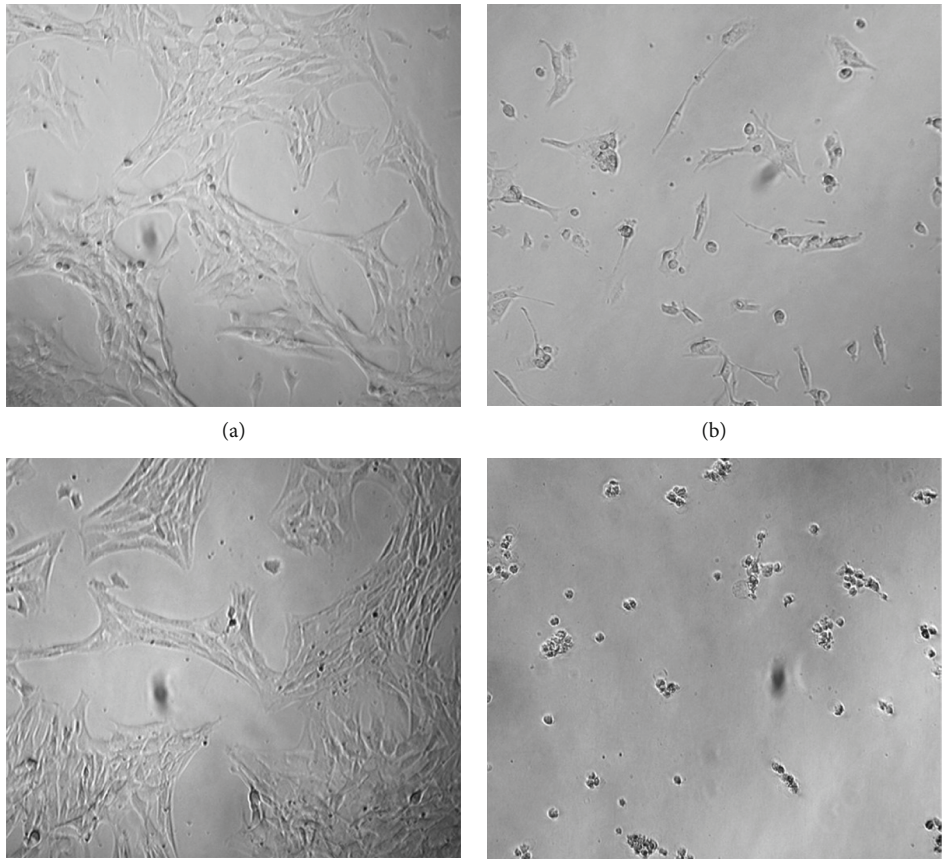

Figure 7: Morphological changes pf PTEN-P2 cells after exposure to plumbagin-loaded nanoemulsions. Bright-field microscopy images show untreated control (a) and cells exposed for 24 h to free plumbagin (4 μmol/l) (b), empty nanoemulsion (c), and plumbagin-loaded (4 μmol/l) nanoemulsion formulation (d).

and narrow polydispersity over extended periods of time, indicating good shelf-life with undemanding temperature requirements that would facilitate manufacturing, storage, and distribution.

Retention of nanoparticle size after dispersion in water or media emulating physiological fluids also suggested potential stability in the gastrointestinal tract that would permit optimal absorption in vivo.

It is reasonably expected that plumbagin delivered/released from the nanoemulsion formulation should exhibit its pharmacological activities towards various types of cancer cells as extensively described in literature, since plumbagin has shown no discrimination toward cancer types. Noteworthy, observed cytotoxicity of plumbagin toward PTEN-P2 prostate cancer cells, both as a free drug and in the form of drug-loaded nanoemulsion, was slightly higher compared to standard of care for prostate cancer—docetaxel or other anticancer drugs in development, rapamycin and 17-AAG encapsulated in the poly(ethylene glycol)-block-poly(D,L-lactic acid) (PEG-b-PLA) micelles [34].

In vitro cytotoxicity studies of the plumbagin-loaded nanoemulsion showed a slightly augmented antiproliferative effect of the active pharmaceutical ingredient (plumbagin) against PTEN-P2 prostate cancer cells compared to free drug. This is not attributable to an additive effect of cytotoxicity of individual components, because the control formulation (nanoemulsion without drug) did not exhibit cytotoxicity at equivalent doses. Thus, increased cytotoxicity of the plumbagin nanoemulsion formulation compared to free plumbagin can be attributed to a higher cellular uptake of the nanoparticulate form, to a stabilizing effect of the nanoemulsion on plumbagin, or a combination of both. Indeed, the high particle surface area [15] associated with a high surface to mass ratio due to the small diameter of the nanoparticulate dispersion is expected to increase its interaction with cell surfaces, yielding a higher cellular uptake of plumbagin. Importantly, plumbagin is an α, β-unsaturated diketone (1,4-naphthoquinone) electrophile capable of undergoing Michael's addition reaction with endogenous nucleophiles [35], including serum albumin [26], and is therefore extremely reactive. Encapsulation of plumbagin in the hydrophobic core of the nanoemulsion would decrease its interaction with nucleophiles before reaching cellular targets. This effect is expected to improve the pharmacokinetic/pharmacodynamic profile of plumbagin when administered in the form of a nanoemulsion.

5. Conclusion

We have developed a plumbagin-loaded nanoemulsion formulation using lipid-based excipients that are biocompatible and have been used in clinically approved pharmaceutical products. High loading capacity and retention of nanoparticle size over extended time and in a simulated physiological environment as well as *in vitro* anticancer activity indicate that the Capryol 90-based nanoemulsion formulation of plumbagin has significant translational potential.

Data Availability

All data are available at Vaccine Research Institute of San Diego, San Diego Science Center, San Diego, CA.

Ethical Approval

Pellficure Pharmaceuticals, Inc., had the final approval of the manuscript.

Disclosure

Neither the funding agency nor any outside organization has participated in study design.

Conflicts of Interest

Borgstrom P, Chrastina A, and Baron VT are inventors on a patent in relation to this work. Borgstrom P is also the CEO of Pellficure Pharmaceuticals, Inc.

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Research Article

Hepatoprotective Screening of *Seriphidium kurramense* (Qazilb.) Y.R. Ling

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Investigation on medicinal plants' therapeutic potential has gained substantial importance in the discovery of novel effective and safe therapeutic agents. The present study is aimed at investigating the hepatoprotective potential of *Seriphidium kurramense* methanolic extract (SKM) against carbon tetrachloride- (CCl_4-) induced hepatotoxicity in rats. *S. kurramense* is one of the most imperative plants for its various pharmacological activities. Therefore, this study was aimed at evaluating the hepatoprotective potential against CCl_4-induced liver toxicity. The serum samples were analyzed for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) together with the oxidative stress mediator levels as nitric oxide (NO), malondialdehyde (MDA), glutathione (GSH), reduced glutathione (GSH), and superoxide dismutase (SOD) as well as peroxidation and H_2O_2 activity. CCl_4 administration resulted in an elevated free radical generation, altered liver marker (AST and ALT) enzymes, reduced antioxidant enzyme, and increased DNA damage. Methanolic extract of *S. kurramense* decreased CCl_4-induced hepatotoxicity by increasing the antioxidant status and reducing H_2O_2 and nitrate content generation as well as reducing DNA damage. Additionally, SKM reversed the morphological alterations induced by CCl_4 in the SKM-treated groups. These results demonstrated that SKM displayed hepatoprotective activity against CCl_4-induced hepatic damage in experimental rats.

1. Introduction

Seriphidium kurramense (Qazilb) Y.R. Ling is an important medicinal and economic plant of the family Asteraceae and endemic to the tribal district, Upper Kurram, Pakistan, and also transported to different parts of the country for the extraction of new drugs [1]. By surveying the literature, it has been confirmed that several medicinal plants of Pakistan collected from Khyber Pakhtunkhwa, including tribal districts, have been screened out. The earlier studies showed that *S. kurramense* was revealed to have the highest medicinal level and also used for insecticide purposes [1, 2]; Kafeel et al., 2018; [2], by clarifying the mechanism underlying the phytochemicals and biological activities of *S. kurramense* and providing a reference to address protective potential. Carbon tetrachloride (CCl_4) is a well-renowned industrial solvent known to cause hepatotoxicity. Free radicals derived from CCl_4 are involved in covalent binding to the macromolecules, which also cause lipid peroxidation [3, 4]. It has also been used as a dry-cleaning agent in industries, as a catalyst in polymer reactions, as a solvent in cleaning metals, and as granule fumigant [5, 6]. Numerous studies have been conducted on CCl_4, which showed toxicity in various pathophysiological conditions [7]. Different studies have shown that CCl_4 intoxication is constrained to the liver and causes oxidative damage to the tissues of the lung, kidney, heart, brain, testis, and blood [8, 9]. Due to succeeding CCl_4 exposure, lipid peroxide and protein carbonyl levels were identified in tissues isolated from the lung, kidney, and testis of rats [10]. CCl_4 requires cytochrome P450 (phase I system) for the activation of metabolic system in the liver to initiate reactive radical species such as proxy trichloromethyl ($^{\cdot}OOCCl_3$) and trichloro-4 methyl ($^{\cdot}CCl_3$), which are involved in increasing lipid peroxidation and protein oxidation causing liver damage [11]. Both radicals are further involved in the initiation of alkoxy ($RO^{\cdot}$) and peroxy ($ROO^{\cdot}$) radicals through their action on the polyunsaturated fatty acids [12]. The silymarin extract obtained from the *Silybum marianum* is a mixture of polyphenols and flavonoids. Commercially prepared silymarin consists of various flavonoids, such as silidianin and silichristin, isosilibinin (isosilybin A and B), and silibinin (silybin A and B). Silibinin is the key component of this mixture. Silymarin has been found to show antioxidant potential and stabilize the membrane; it also inhibits fibrogenesis, reduces inflammatory reaction, and provokes hepatocyte regeneration. These results were verified through several clinical trials [13–16]. Therefore, the present study was designed with the aim to evaluate their biological activities including hepatoprotective potential of *Seriphidium kurramense* methanol extract (SKM) against CCl_4-induced hepatotoxicity in rats. Our results showed that SKM treatment significantly abolished CCl4-induced hepatotoxicity via attenuating free radical generation, boosting antioxidants, and preventing DNA damage.

2. Results

2.1. Effects of SKM on Liver Biomarker Indices. The ALT and AST activities and globulin levels were significantly elevated, while the level of albumin and total protein was decreased in the CCl_4-treated rats compared to the control group (Table 1). However, the SKM administration reverses CCl_4 effects by reducing ALT and AST activities and the altered level of albumin, globulin, and total protein. Notably, the SKM+CCl_4-treated groups showed results comparable to the group treated with silymarin. Interestingly, SKM effects on the liver biomarkers (ALT, AST, albumin, globulin, and total protein) were recorded to be dose-dependent (Table 1).

2.2. The Effects of SKM on the Activities of CAT, POD, and SOD and the Level of GSH. Table 2 shows the effect of SKM on the activities of antioxidant parameters in experimental animals subjected to CCl_4 toxicity. The antioxidant enzyme (CAT, POD, and SOD) activities and the level of GSH were significantly increased in the CCl_4-treated rats administered with SKM compared to rats treated with CCl4 only. The administration of SKM to CCl_4-treated rats showed a dose-dependent increase in the activities of CAT, POD, and SOD and the level of GSH. Consistently, the increased antioxidant indices (CAT, POD, SOD, and GSH) were greater in the group treated with SKM only compared to the groups treated with both SKM+CCl_4 as well as the control groups (normal control and silymarin group).

2.3. The Effect of SKM on the Level of TBARS, Nitrite, and H_2O_2. The level of TBARs, nitrite, and H_2O_2 was recorded to be significantly elevated in the group treated with CCl_4 only compared to the groups treated with the SKM and silymarin as well as the control group (Table 3). The elevation recorded of these markers of oxidative stress in the groups treated with CCl_4 only was reduced in a dose-dependent manner after SKM administration. The results obtained in the CCl_4 groups administered with SKM were comparable to the CCl_4 group treated with silymarin.

2.4. SKM Treatment Attenuated CCl_4-Induced DNA Injury. The comet and tail length, DNA in the tail, and tail moments were significantly high, while the head length and DNA in the head were significantly low in CCl_4-treated rats than in the control group (Figure 1, Table 4). Nevertheless, the administration of SKM to the groups treated with CCl_4 ameliorated the DNA damage and was comparable with the group treated with both CCl_4 and silymarin. Interestingly, the group treated with solo SKM showed better results compared to the groups treated with both CC_{l4} and SKM (Figure 1, Table 4).

2.5. Defensive Effect of SKM on Histoarchitecture of the Liver. Hematoxylin and eosin are used to stain the thinly sliced sections of liver tissue which were microscopically photographed at 40x to examine various morphological alterations, as shown in Figure 2. Normal morphology is shown in the control group with the distinctive central vein, Kupffer cells, hepatocytes, and sinusoids (Figure 2). Furthermore, CCl_4 treatment caused a noticeable elevation in fatty changes, cellular hypertrophy, inflammatory cell infiltrations, ballooning, and dilation of the central vein in the liver tissues. However, SKM administration (150 mg/kg) presented the hepatic structure with little fatty changes, dilation

Table 1: Effect of *S. kurramense* on liver biomarkers.

Treatment	ALT (mg/dL)	AST (mg/dL)	Albumin (mg/dL)	Globulin (mg/dL)	Tissue protein (μg/mg tissue)
Control (normal)	38 ± 2.16^{e}	42 ± 2.16^{d}	4.47 ± 0.59^{a}	3.69 ± 0.38^{b}	3.26 ± 0.23^{a}
DMSO+olive oil	37 ± 2.16^{e}	43 ± 2.16^{d}	4.33 ± 0.81^{a}	3.69 ± 0.42^{b}	3.25 ± 0.32^{a}
CCl_4 (1 mL/kg)	109 ± 4.19^{a}	106 ± 4.11^{a}	1.95 ± 0.29^{d}	4.07 ± 0.36^{a}	1.37 ± 0.16^{c}
CCl_4+silymarin	96 ± 3.36^{b}	86 ± 4.61^{b}	3.07 ± 0.37^{b}	3.94 ± 0.54^{b}	2.67 ± 0.33^{b}
CCl_4+SKM (150 mg/mg)	86 ± 4.42^{c}	84 ± 4.42^{b}	2.46 ± 0.14^{c}	3.17 ± 0.66^{b}	1.62 ± 0.42^{c}
CCl_4+SKM (300 mg/kg)	72 ± 3.49^{d}	58 ± 3.49^{c}	3.14 ± 0.21^{b}	3.89 ± 0.19^{b}	2.75 ± 0.45^{b}
SKM (150 mg/kg)	38 ± 2.16^{e}	44 ± 2.16^{d}	4.14 ± 0.44^{a}	3.59 ± 0.31^{b}	3.41 ± 0.42^{a}
SKM (300 mg/kg)	39 ± 2.16^{e}	42 ± 2.16^{d}	4.22 ± 0.50^{a}	3.62 ± 0.46^{b}	3.31 ± 0.32^{a}

Values are expressed as mean ± SD (7). Values with different alphabet letters down the column indicate a significant difference ($p < 0.05$). SKM: *Seriphidium Kurramense* methanol extract.

Table 2: Effect of *S. Kurramense* on antioxidant parameters.

Treatment	CAT (U/min)	POD (U/min)	SOD (U/mg protein)	GSH (μM/g tissue)
Control (normal)	7.3 ± 0.82^{a}	9.38 ± 1.2^{a}	5.33 ± 0.75^{a}	22.46 ± 1.32^{a}
DMSO+olive oil	7.2 ± 0.78^{a}	9.32 ± 1.17^{a}	5.23 ± 0.86^{a}	22.41 ± 1.36^{a}
CCl_4 (1 mL/kg)	2.36 ± 0.31^{d}	3.01 ± 0.33^{d}	2.03 ± 0.42^{d}	6.42 ± 0.72^{d}
CCl_4+silymarin	5.4 ± 0.56^{b}	8.1 ± 1.12^{b}	4.12 ± 0.96^{b}	18.42 ± 2.16^{b}
CCl_4+SKM (150 mg/mg)	4.6 ± 0.68^{c}	6.23 ± 0.85^{c}	3.78 ± 0.42^{c}	14.56 ± 1.11^{c}
CCl_4+SKM (300 mg/kg)	5.9 ± 0.65^{b}	8.52 ± 1.23^{b}	4.36 ± 0.76^{b}	17.23 ± 1.26^{b}
SKM (150 mg/kg)	7.3 ± 0.72^{a}	9.28 ± 1.09^{a}	5.28 ± 0.62^{a}	22.32 ± 1.06^{a}
SKM (300 mg/kg)	7.1 ± 0.93^{a}	9.10 ± 1.11^{a}	5.24 ± 0.72^{a}	22.42 ± 1.42^{a}

SKM: *Seriphidium kurramense* methanol extract. Values expressed as mean ± SD (7). Means with different alphabet letters within the column indicate a significant difference ($p < 0.05$).

Table 3: Effect of SKM on TBARS, nitrite, and H_2O_2 in CCl_4-treated rats.

Treatment	TBARS (nM/min/mg protein)	Nitrite (μM/mL)	H_2O_2 (μM/mL)
Control (normal)	24.56 ± 2.16^{a}	49.24 ± 2.04^{d}	0.39 ± 0.09^{e}
DMSO+olive oil	24.47 ± 2.32^{a}	49.23 ± 2.25^{d}	0.38 ± 0.08^{e}
CCl_4 (1 mL/kg)	47.22 ± 3.17^{b}	87.68 ± 4.22^{a}	1.03 ± 0.26^{a}
CCl_4+silymarin	28.23 ± 1.42^{c}	56.23 ± 2.17^{c}	0.52 ± 0.11^{c}
CCl_4+SKM (150 mg/mg)	30.26 ± 2.42^{c}	63.01 ± 2.23^{b}	0.75 ± 0.15^{b}
CCl_4+SKM (300 mg/kg)	29.43 ± 2.17^{c}	58.23 ± 3.32^{c}	0.48 ± 0.08^{c}
SKM (150 mg/kg)	24.24 ± 1.32^{a}	47.67 ± 2.31^{d}	0.38 ± 0.09^{e}
SKM (300 mg/kg)	23.78 ± 2.36^{a}	48.03 ± 2.09^{d}	0.39 ± 0.11^{e}

SKM: *Seriphidium kurramense* methanol extract. Values expressed as mean ± SD (7). Means with different alphabet letters within the column indicate a significant difference ($p < 0.05$).

of the blood vessel, and uniform morphology of hepatocytes similar to the control group. Similarly, silymarin (50 mg/kg) attenuated the cellular alterations and distractions as expressed. The hepatic histology illustrated that SKM was a higher defensive dose (300 mg/kg) of SKM.

3. Discussion

Carbon tetrachloride (CCl_4) is a well-known lethal hepatotoxin, and free radical production causes different disorders [17–19]. For centuries, experimental models have been used to investigate mechanisms of oxidant/free radical toxicity in induced chronic disorders [20]. The current study was conceded out to evaluate the ameliorative potential of *S. kurramense* against liver damage in CCl_4-treated experimental rats. Our result corroborates with Singh et al. [21], who established the antioxidative properties of *Solanum xanthocarpum* fruit extract against drug-induced toxicity.

CCl_4 induces oxidative stress by free radical generation, causes tissue injury by DNA damage, distressed enzymatic level, and elevated lipid peroxidation. Previously, it has been reported that CCl_4 (1 mL/kg) administration for 4 weeks at alternating days caused hepatic fibrogenesis, injured the functional reliability of cell membrane and hepatic mitochondrial function, and increased serum enzymes and endogenous antioxidant enzyme pool [22, 23]. Recent studies reveal that CC4-treated rats showed a high intensity of liver markers ALT, AST, and ALP in the serum due to

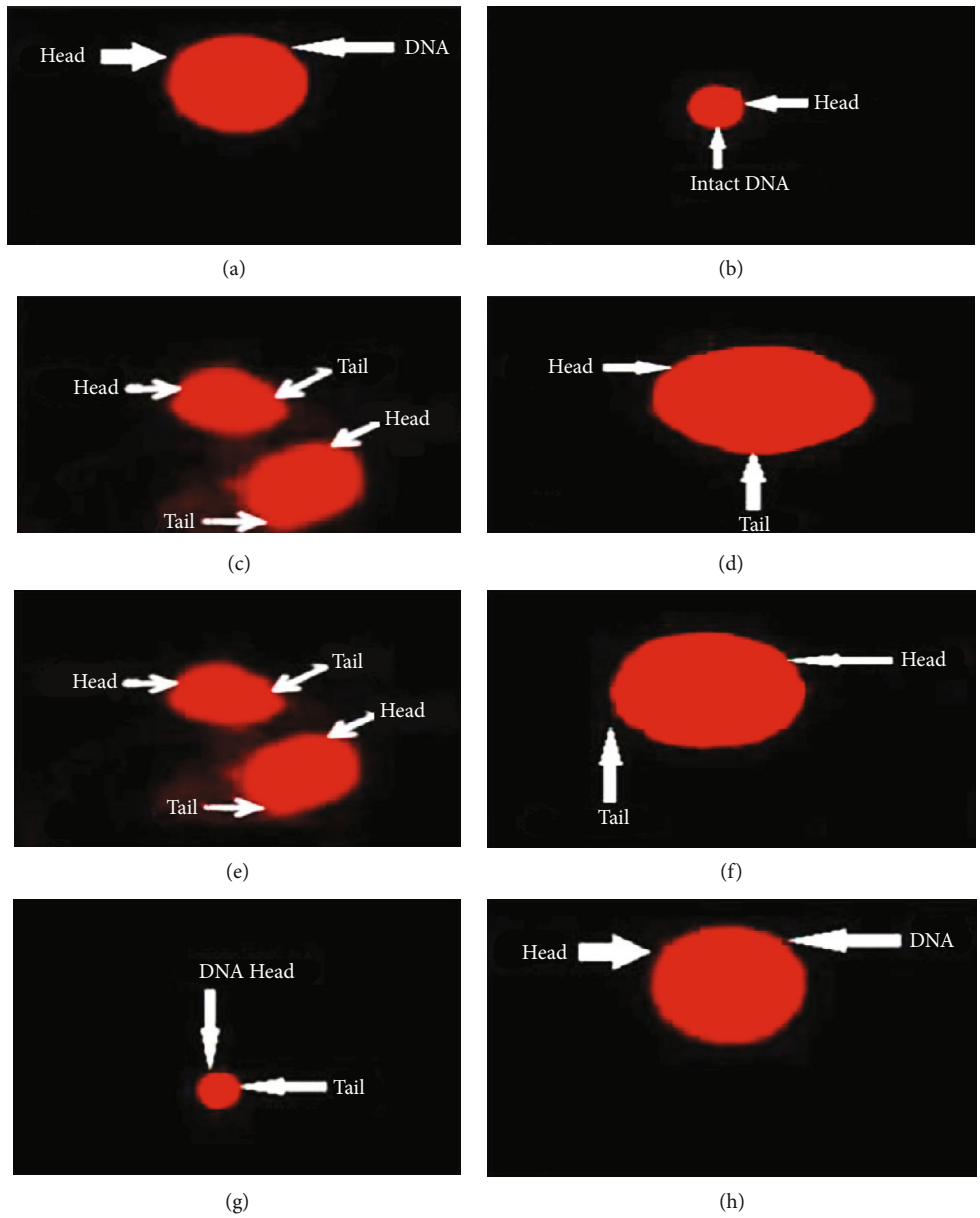

Figure 1: The fluorescence photomicrograph of *Seriphidium kurramense* methanol extract effect on DNA of hepatic cells: (a) control group, (b) vehicle control, (c) CCl_4 only, (d) CCl_4+rutin, (e) CCl_4+low dose (150 mg/kg), (f) CCl_4+high dose (300 mg/kg), (g) low dose alone, and (h) high dose alone.

damaged hepatocellular membrane reliability [12, 24]. In the present study, the plant's methanol extract showed its protective aptitude according to its dose concentration. Moreover, the low dose (150 mg/kg) was protective, while the protective effect of the high dose (300 mg/kg) was significantly incoherent with silymarin. Our results are in accordance with the study of Sahreen et al. [12], in which they documented that the antifibrotic effects are caused by the antioxidant activity of *Rumex hastatus* D. On the other hand, serum activities of AST, ALT, and ALP are related to hepatocyte membrane damage, leaked out into plasma due to the damaged membrane. Singh et al. [25] suggested that the above serum markers' high levels can be the consequence of massive centrilobular necrosis and cellular infiltration in the liver.

Nevin and Vijayammal [8] proposed that compounds and chemicals' toxicological nature can be regularly tested by total protein estimation. Consistent with previous findings herein, CCl_4 intoxication reduced protein levels both in the serum and at the tissue level, and SKM treatment reversed these CCl_4-induced effects. Furthermore, a high dose of SKM showed more promising and protective effects than a low dose against CCl_4-induced protein level dysregulation, suggesting a dose-dependent action of SKM [26]. These results showed that *S. kurramense* is a worthy candidate to inhibit the DNA damage in renal tissues. However, the antioxidant resistance system includes the enzymatic antioxidants (CAT, POD, SOD, GST, and GSH), which contributed a fundamental role in the protective system *via* scavenging free radicals. It has been reported that CCl_4

Table 4: Effects of SKM on the genotoxic parameters in CCl_4-treated rats.

Treatment	Comet length (μm)	Head length (μm)	Tail length (μm)	% DNA in the head	% DNA in the tail	Tail moment (μm)
Control (normal)	62.14 ± 3.6^{d}	55 ± 3.03^{a}	7.3 ± 1.11^{d}	91 ± 3.03^{a}	9.01 ± 1.16^{d}	31.51 ± 2.01^{c}
DMSO+olive oil	62.23 ± 3.4^{d}	55.5 ± 3.2^{a}	7.5 ± 1.02^{d}	90 ± 3.45^{a}	9.86 ± 1.37^{d}	31.42 ± 2.12^{c}
CCl_4 (1 mL/kg)	86.21 ± 4.2^{a}	44 ± 3.33^{c}	42 ± 2.11^{a}	65 ± 2.12^{d}	35 ± 2.56^{a}	41.87 ± 2.07^{a}
CCl_4+silymarin	65.27 ± 3.1^{c}	52.3 ± 3.1^{b}	13.9 ± 1.23^{c}	86 ± 3.16^{b}	14.1 ± 1.9^{c}	32.42 ± 2.01^{c}
CCl_4+SKM (150 mg/mg)	73.03 ± 3.3^{b}	45 ± 3.42^{c}	19 ± 2.45^{b}	71.3 ± 3.1^{c}	29.5 ± 2.3^{b}	36.56 ± 2.02^{b}
CCl_4+SKM (300 mg/kg)	65.78 ± 3.3^{c}	45.2 ± 2.3^{c}	20.58 ± 1.4^{b}	87.1 ± 3.2^{b}	12.87 ± 1.1^{c}	32.54 ± 2.04^{c}
SKM (150 mg/kg)	61.62 ± 1.6^{d}	54.3 ± 2.4a	7.31 ± 1.9d	91.2 ± 3.1a	9.78 ± 1.5d	30.50 ± 2.02c
SKM (300 mg/kg)	62.23 ± 1.3^{d}	55.6 ± 2.2^{a}	7.63 ± 1.01^{d}	90.1 ± 4.1^{a}	9.71 ± 1.13^{d}	30.47 ± 2.04^{c}

SKM: *Seriphidium kurramense* methanol extract. Values expressed as mean ± SD (7). Means with different alphabet letters within the column indicate a significant difference ($p < 0.05$).

treatment can cause a significant reduction in the CAT, POD, SOD, GST, and GSH levels [27, 28]. Interestingly, the level of GSH reduced because of its more utilization by the hepatocytes in hunting toxic radicals produced by CCl_4. [29] also documented the decline in levels of all enzymes and GSH content in liver tissue by CCl_4 supervision. However, SKM treatment enhanced antioxidant enzymes, including CAT, SOD, POD, GST, and GSH level, and decreased under CCl_4-induced stress condition [29] using *Sonchus asper* as a medicinal plant. Furthermore, CCl_4 was also showing its toxic belongings by changing the levels of TBARS, H_2O_2, tissue protein, and nitrite contents. Peroxidation of lipids provoked overexpression of genetic fibrogenic cytokines by motivating the collagen amalgamation and activating hepatic [30]. Herein, in agreement with previous findings: our results demonstrated reduced protein level and enhanced TBARs, H_2O_2, and nitrite content upon CCl_4 administration. However, SKM treatment significantly recovered CCl_4-induced toxicity by reducing TBARs level, H_2O_2 concentration, and nitrite content, likewise the silymarin-treated group. Furthermore, the high-dose treatment of SKM was more effective than the low dose.

Liver regeneration has a significant role in the resistance against chemical-induced damage [31], and its histopathological analysis is the shortest way of evaluating the toxic effect of a drug such as CCl_4 and extract of different plants. In our results, liver histological analysis showed a high degree of liver cell damage, fibrosis, necrosis, cellular hypertrophy, and central lobule disruption in the CCl_4-treated group compared to the normal subjects; a comparable result has been demonstrated previously by Chen et al. [32]. CCl_4-caused oxidative damage to DNA in the mammalian cells has been pragmatic [33]. The single-stranded or double-stranded break in the DNA is because of free radicals, injuring DNA integrity [34, 35]. Our single-cell gel electrophoresis results showed the extent of DNA damage in the CCl_4-treated group compared to the normal subject [36].

Furthermore, an increase in comet tail moment and decline in DNA percentage were observed in CCl_4-treated rats compared to the control group. Akram et al. [37] used comet assay to report DNA damage in the rats' ovaries upon sodium arsenate treatment. Sodium arsenate treatment concentration depends on increased oxidative stress in the tissues, leading to abnormal oocytes with damaged DNA. And also, it is recommended to study the advanced techniques using SEM analysis for future study [38, 39]. However, in the present investigation, SKM treatment showed an increase in comet head length, DNA percentage, and reduction of comet length, tail length, and moment, as well as DNA percentage in the tail of the comet, suggesting its protecting effects against CCl4-induced cellular toxicity by preventing DNA damage. Notably, a high dose of SKM was more prominent and significant than a low dose.

4. Materials and Methods

4.1. Plant Material and Extraction. *Seriphidium kurramense* was collected during the spring season in 2018 from Parachinar tribal district, Khyber Pakhtunkhwa (KPK), Pakistan. Dr. Mushtaq Ahmad, Professor at Quaid-i-Azam University Islamabad, identified the plant specimen. Aerial parts of *S. kurramense* were dried under shade and ground to fine powder, and about 2 kg was pulverized using an electric blender. The finely powdered sample was macerated in 4 L methanol for two weeks at room temperature. Extract of the plant was completed twice to get their soluble portions using resultant filtrate. Subsequent to obtaining cured methanol extract, particular fraction was ended by the rotary evaporator [40, 41]. Afterward, the plant extract was dried out and kept at 4°C for further explorations, while for *in vivo* analysis, CCl_4 was preferred to rouse toxicity in the liver of *Sprague Dawley* rats as an animal model and to scrutinize them at molecular, biochemical, and tissue level. The mixture was evaporated to dryness; the slurry extract was dried completely at -70°C. The mechanism is shown in Figure 3.

4.2. Preparation of CCl_4 and SKM. CCl_4 was prepared using olive oil as a vehicle. CCl_4 was added to the olive oil in the ratio of 30 : 70 *w*/*w*. The intraperitoneal injection of CCl_4 mixed with olive oil was carried out for four weeks. Simultaneously, the plant methanol extract was administered at a dose of 150 and 300 mg/kg body weight. Silymarin was used as the reference drug at a dose of 100 mg/kg body weight. The various doses of SKM were prepared in DMSO as the vehicle for the plant extract.

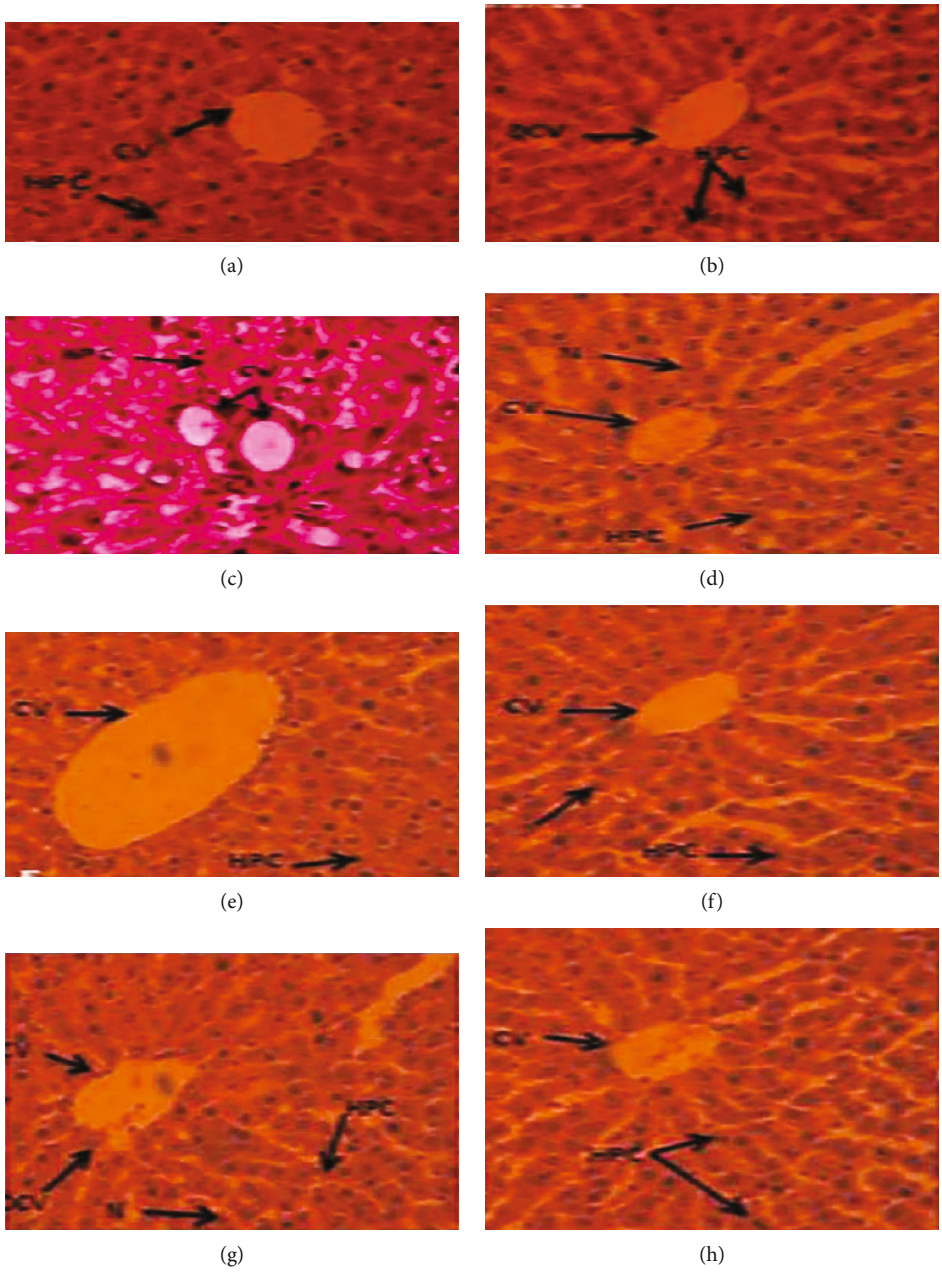

Figure 2: Liver histopathological observations of control and treated groups at 40x: (a) control, (b) vehicle control, (c) CCl_4 only, (d) CCl_4 +silymarin 200 mg/kg, (e) CCl_4+SKM 150 mg/kg, (f) CCl_4+SMM 300 mg/kg, (g) SKM 150 mg/kg, and (h) SKM 300 mg/kg.

4.3. Experiment. Fifty-six rats with 150-200 g weight were kept at the primate facility in Quaid-i-Azam University, Islamabad, Pakistan. The experimental animals were subjected to 12 h dark and light cycle, fed on standard rat feeds, and provided with water *ad libitum*. The methods for animal handling were following the institutional ethical committee's guidelines on scientific research. According to Table 5, the experimental animals were divided into eight groups (each $n = 6$). Group I served as a control, group II contained rats that were treated with DMSO (10%, 1 mL/kg), group III contained rats treated with CCl_4 (i.p) (1 mL/kg), group IV received CCl_4+silymarin (100 mg/mL/kg) (as a reference group), group V rats received CCl_4+SKM (150 mg/kg, orally), group VI received CCl_4+SKM (300 mg/kg, orally), group VII received SKM (150 mg/kg orally), and group VIII received SKM (300 mg/kg orally).

4.4. Collection of Blood Sample and Isolation of Organs. The experimental rats were subjected to chloroform anesthesia. The jugular vein of the unconscious animal was cut using a sharp disserting blade. The blood samples were collected into clean plain sample bottles. The blood samples were centrifuged for 15 min at 10,000 rpm. After centrifugation, the serum samples were collected and stored in appropriate sample bottles. The animals were dissected, and part of the liver organ was collected, cleaned, and stored in liquid

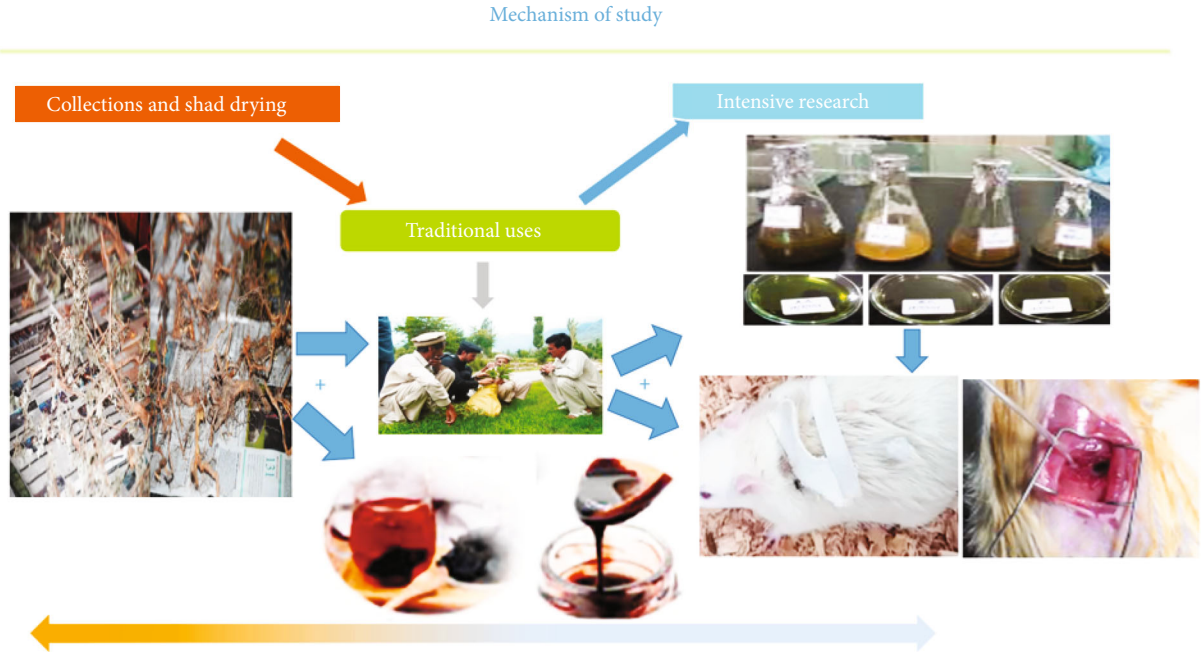

Figure 3: Mechanism of Seriphidium kurramense from traditional medicine to in vivo analysis.

Table 5: Distribution of animal groups (each containing 6 rats).

Group (control)	Given no treatment
Group 1 (control normal)	Normal healthy feeding
Group 2 DMSO+olive oil	Given 10% DMSO in olive oil orally (1 mL/kg rat body weight)
Group 3 (CCl_4)	Given 30% CCl_4 in olive oil i.p (1 mL/kg rat body weight)
Group 4 (silymirin+CCl_4)	Given 30% CCl_4 in olive oil i.p (1 mL/kg rat body weight)+silymarin (100 mg/mL in DMSO) orally
Group 5 (low dose+CCl_4)	Given 30% CCl_4 in olive oil i.p (1 mL/kg rat bodyweight)+*S. kurramense* methanol (SKM) (150 mg/kg rat body weight) orally
Group 6 (high dose+CCl_4)	Given 30% CCl_4 in olive oil i.p (1 mL/kg rat bodyweight)+*S. kurramense* methanol (SKM) (300 mg/kg rat body weight) orally
Group 7 (SKM)	Given only *S. kurramense* methanol (SKM) (150 mg/kg rat body weight) in DMSO orally
Group 8 (SKM)	Given only *S. kurramense* methanol (SKM) (300 mg/kg rat body weight) in DMSO orally

nitrogen at 70°C for analysis of tissue homogenates. Following homogenization, the liver homogenate was centrifuged, and the supernatant was collected and was preserved in phosphate buffer formalin 10%, for histopathological observations.

4.5. Biochemical Analysis. ALT (alanine aminotransferase estimation), AST (aspartate aminotransferase estimation), ALP (alkaline phosphatase estimation), and albumin were estimated using diagnostic tools.

4.5.1. Protein Estimation. The full soluble hepatic proteins were anticipated by the technique of Lowry et al. [42]. An amount of 80 mg of tissue was weighed of every organ and homogenized within the phosphate buffer. The organ tissues were centrifuged after homogenization at 10,000 rpm at 4°C for 20 min. The 0.1 mL sample is mixed in 1 mL basic solution and incubated for 10 min; then, Folin-Ciocalteu reagent was added to each tube with the ratio of 1 : 1 (v/v) and vortexed. The absorbance was recorded at 595 nm after 30 min incubation. BSA standard curve was used to determine soluble protein concentration.

4.5.2. Globulin Estimation. Globulin evaluation was carried out using the following formula:total protein – albumin.

4.5.3. Assessment of Antioxidative Profile

(1) Catalase Assay (CAT). Catalase action was intended by Chance and Maehly's [42] technique with some changes. For the catalase evaluation, the reaction fusion restricted 2500 μL of 50 mM phosphate buffer (pH 7.2), hydrogen peroxide 420 μL (5.9 mM), and extract of enzyme (100 μL). After 1 min, absorbance alteration of the reaction mixture of the solution was recorded at 240 nm. One entity, catalase activity, was distinct, since at 0.01 units per minute, there is an absorbance change.

(2) Peroxidase Assay (POD). POD assay was resolved by Kakkar et al.'s [4] technique. The reactants of peroxidase

assay were 1000 μL enzyme extract, 100 μL guaiacol (20 mM), 300 μL H_2O_2 (40 mM), and 2500 μL 50 mM phosphate buffer (pH 6.8). The absorbance change of the reaction solution was measured after 1 min at 470 nm. A change of absorbance of 0.01 units per minute's peroxidase activity was observed.

(3) Superoxide Dismutase Assay (SOD). SOD assay was performed using Spitz and Larry's [43] technique. This process was initiated by using 100 μL reaction mixture of 186 μM PNS (phenazine methosulphate), 1200 μL sodium pyrophosphate (0.052 mM; pH =7.0), and supernatant 300 μL derived from the homogenate of the liver, which was integrated into the reaction mixture. The entire mixture was subjected to centrifugation for 10 min at 1500 rpm and after that at 10000 rpm for 15 min. The enzyme's reaction started with the addition of 0.2 mL of 780 μM NADH and followed by the addition of 1000 μL glacial acetic acid. The intensity of color estimated the amount of chromogen produced at an absorbance of 560 nm, and the results were recorded in units per milligram.

(4) Glutathione S-Transferase Assay (GST). To evaluate GST, Habig et al.'s [44] method was used. The summary of all the reactants for this test includes 200 μL of reduced glutathione of 1 mM, 1475 μL phosphate buffer of 0.1 M (pH = 6.5), and enzyme extract of 300 μL, in a total volume of 2000 μL. At the absorbance of 340 nm, a change was observed. The activity of enzymes was assessed throughout with the formation of *M* conjugate per minute. The extermination molarity, 9.6×103 M^{-1} cm^{-1}, was used as a coefficient here.

(5) Reduced Glutathione Assay (GSH). The assay was performed using Jollow et al.'s [45] protocol. The homogeneous mixture of the sample contained 1000 μL of 4% sulfosalicylic acid. The appetizer was kept at 4°C for 1 h and centrifuged for the next 20 min at 4°C (1200 g). The entire 3000 μL volume of the reactant mixture included 100 μL clean aliquot, 200 μL DTNB (100 mM), and 2700 μL phosphate buffer (0.1 M; pH = 7.4). The color (yellow) variation at various combinations of the reactants was calculated instantly by maintaining 412 nm of the absorbance. Reduced glutathione action was articulated as micromolar GSH per gram tissue piece.

(6) Lipid Peroxidation (TBARS). This original assay was conducted using Iqbal and Wright's [46] method. The whole 1000 μL of the main reacting sample included 0.1 M phosphate buffer (580 μL) maintained at pH 7.4, homogenate sample (200 μL), the 100 mM of ascorbic acid (200 μL), and 100 mM ferric chloride (20 μL). The whole reacting sample was kept at 37°C for 1 h in the incubator. The 1000 μL of 10% trichloroacetic acid was added to block the reaction. The sample tubes were inserted in hot water maintained at 100°C, followed by adding 0.67% thiobarbituric (1000 μL) for 20 min, and next positioned on the compressed ice-bath, and then, centrifugation for 10 min ($2500 \times$ g) was started. The amount of TBARS (lipid peroxidation) in every sample was premeditated by captivating the absorbance of supernatant against a blank reagent on a spectrophotometer at 535 nm. The outcomes were uttered using 1.56×105 M^{-1} cm^{-1} molar destruction coefficient for TBARS per minute per milligram tissues at 37°C.

(7) Hydrogen Peroxide Assay (H_2O_2). H_2O_2 activity was executed following the Pick and Keisari's [47] protocol through umpired horseradish peroxidase-reliant oxidation of phenol red. The main constituents included 0.5 M phosphate buffer (pH = 7.0), 8.5 units of horseradish peroxidase, 5.5 nM dextrose, and 2 mL of a homogenate of tissue (hanging in 1000 μL of a solution of 0.28 nM phenol red), and the whole mixture of the sample was incubated for 60 min at 37°C followed by addition of 10 μL of 1 N sodium hydroxide to cease further reaction. The centrifugation was performed at $800 \times$ g for 5 min. At the wavelength of 610 nm, the supernatant's absorbance against the blank reagent was calculated. The nM H_2O_2 was fed in milligram per minute per tissue using standard curve H_2O_2 oxidized phenol red.

(8) Nitrite Assay. Grisham et al. [48] experimented to authenticate the nitrite assess. The Griess reagent was used in this experiment. The sample (100 mg tissue homogenate) was deproteinized with 5% $ZnSO_4$ and 0.3 M NaOH (100 μL). The entire mixture was subjected to 15-20 min of centrifugation at $6400 \times$ g. A volume of 1 mL Griess reagent along with 20 μL supernatant was added in the cuvette. The absorbance of the reaction mixture was recorded at 540 nm. The curve of $NaNO_2$ was used for evaluating the quantity of nitrite in samples.

(9) Histopathological Study of Tissues. The paraffin-rooted bruising route finished histopathology of tissue appraisal. The different steps demand obsession with a biological taster in sticky stuff, safeguarding their morphology, and avert the tissue decomposes. Consequently, the new hepatic tissues were segmented into tiny sections and set in 10% formalin. The preset tissues were more soaked, and progression in the way of a mounting succession of 50%, 70%, 90%, and 100% alcohol, heading for the tissues, maintains on a rigid solid medium and, consequently, assists slight segments to be incised. The tissues were safe on tough solid wedges, using paraffin-implanting. Slides were fixed by slicing skinny strata of the fixed-tissue tasters 3-4 μm through staining with eosin and hematoxylin. Finally, the examination of slides was carried out in the light microscope (DIALUX 20 EB) at 10x and 40x and pictures using an HDCE-50B camera were taken.

(10) Comet Assay. To determine the DNA damage, the protocol of Dhawan et al. [29] with some modifications was used.

(11) Lysing Solution. The fusion of solution was made by adding 1.46 g of 2.5 M NaOH, 1.2 g of 100 mM Trizma base, and 37.2 g of 100 mM EDTA into 700 mL distilled water. By the addition of HCl or NaOH, pH was adjusted to 10.

Adjustment of entire volume was made equal to 890 mL through distilled H_2O. DMSO and Triton X increase the volume up to the required level, and then, a solution was placed on standby at room temperature. Later on, 10% DMSO and 1% Triton X were added to result in the lysing solution's final state. The whole solution was kept in the refrigerator for 30 min.

(12) PBS Buffer (Mg^{2+}, Ca^{2+}). It was diluted by 990 mL distilled water, and the final volume reached up to 1000 mL at room temperature and regulated at pH 7.4.

(13) Electrophoresis Buffer. Intended for 1X buffer groundwork in distilled H_2O, 30 mL NaOH was added to 0.5 mL of 200 mM EDTA, and the whole volume was taken up to 1000 mL. At room temperature, pH was fixed at less than 1.

Neutralization buffer: 48.5 g of 0.4 M Tris was mixed with 1 L of distilled water, and conc. HCl was added at room temperature to fixed pH of 7.5.

Staining solution: 30 μg/mL stock solution of 10X ethidium bromide. Ten milligrams of ethidium bromide was added to 50 mL dist. H_2O to obtain ethidium bromide solution. This solution was used as a staining agent. A.0.5% of low melting point agarose (LMPA) was all set through the amalgamation of 250 mg of low melting point agarose in 1% PBS solution (50 mL). Refrigeration was stopped, and temperature was stabilized at 37°C by putting in water bath earlier to use a 1% normal melting agarose (NMA). This solution resulted by adding 500 mg of NMA in 50 mL H_2O. Suspension of the gel is formed by heating MA in 50 mL water.

4.6. Preparation of Slides. The NMA and LMPA solutions were all set for the slide preparation, as illustrated earlier. Slides were set *via* sinking in methanol and burn up over a fire to get rid of dirt. The small portion of the slide was sunk in warm NMA agarose and subsequently took away gradually. Dirt-free inferior surface was kept in a dish designed for solidification. The slides were dried in the air, tagged, and placed at ordinary temperature. For the extraction purpose, a minute portion of tissue was placed in a cold solution of HBSS (1 mL) containing DMSO and EDTA in a ratio of 10 : 20. Small pieces of tissues were crushed, followed by the addition of 75 μL LMPA in it. On stored slides, this blend was coated and enclosed by a coverslip. Slides were placed in ice packs for the solidification of gel. After 5-10 min, gradually remove the coverslips and third coat by addition of 80 μL of LMPA on the same slide; using the ice packs, they were made dried. This was further followed by removing the coverslips and putting slides in lysing solution. Sides were protected from the light and chilled for 2 h.

4.7. Electrophoresis. After staying for 2 h in lysing solution, the slides were removed adjusted with a flat gel kit. The newly equipped buffer was dispensed in a gel container, and every slide was placed in the buffer for 20 mins to unzip DNA. At 24 volts, the electric supply was switched on for 30 min. Slides were gradually taken away, and next is the addition of neutralizing buffer. A similar practice was repeated again and again. A 80 μL of 1X ethidium bromide staining was done, and slides were covered with coverslips.

4.8. Slide Visualization. DNA damage was recognized through a fluorescent microscope at 40x. The degree of DNA injury was determined through the software of image investigation CASP 1.2.3. At the same time, approximating the proportion of transferred DNA and also emigrational span was done. Usually, in all tasters, 50 to100 cells were studied. To analyze the relationship between the quantities of per cell migration, numbers of cells with improved migration, feasibility, and emigrational aptitude among injured cells were counted.

5. Conclusion for Future Biology

Seriphidium kurramense possessed potent potential against CCl_4-induced hepatotoxicity by reducing oxidative and nitrosative stress, boosting antioxidant capacities, and reducing cellular toxicity attenuating DNA damage. Moreover, the inspected plant might be measured as an impressive natural cause to widen novel drugs and present a feasible significance of treating various diseases in the mounting world.

Abbreviations

CCl_4: Carbon tetrachloride
H_2O_2: Hydrogen peroxide
$OOCCl_3$: Proxy trichloromethyl
CCl_3: Trichloro-4 methyl
RO: Alkoxy radical
ROO: Peroxy radical
CAT: Catalase assay
POD: Peroxidase assay
SOD: Superoxide dismutase assay
GST: Glutathione S-transferase assay
GSH: Reduced glutathione assay
TBARS: Estimation of lipid peroxidation
PBS: Phosphate-buffered saline
LMPA: Low melting point agarose
NMA: Normal melting agarose
DMSO: Dimethyl sulfoxide
EDTA: Ethylenediaminetetraacetic acid
DNA: Deoxyribonucleic acid
CASP: Critical Appraisal Skills Programme
SKM: *Seriphidium kurramense* methanol extract
ALT: Alanine aminotransferase estimation
AST: Aspartate aminotransferase estimation
ALP: Alkaline phosphatase estimation.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors claim no conflict of interest for the work conducted in this manuscript.

Authors' Contributions

M.A. and H.H. collected plants, performed experiments, collected and analyzed data, and drafted the manuscript. AH supervised the whole experimental work and helped mainly in the write-up. A.R., W.H., and MU have put a lot of their input in the designing and critical writing of the bioactivity part of this work. S.A., Y.S.A., O.B., M.K., A.O., Z.M.A, J.S.-R., and Y.N.M.B. Involved in analysis and editing of paper. M.M.A., S.D.D., M.F.R., and J.S.-R. revised the manuscript and help in arranging data in tables. All authors read the paper and approve it for submission.

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