



Anticancer Chemodiversity of Ranunculaceae Medicinal Plants: Molecular Mechanisms and Functions



Da-Cheng Hao^{1,*}, Chun-Nian He², Jie Shen² and Pei-Gen Xiao^{2,*}

¹Biotechnology Institute, School of Environment and Chemical Engineering, Dalian Jiaotong University, Dalian 116028, China; ²Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, Beijing 100193, China

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Abstract: The buttercup family, *Ranunculaceae*, comprising more than 2,200 species in at least 62 genera, mostly herbs, has long been used in folk medicine and worldwide ethnomedicine since the beginning of human civilization. Various medicinal phytometabolites have been found in *Ranunculaceae* plants, many of which, such as alkaloids, terpenoids, saponins, and polysaccharides, have shown anti-cancer activities *in vitro* and *in vivo*. Most concerns have been raised for two epiphany molecules, the monoterpene thymoquinone and the isoquinoline alkaloid berberine. At least 17 genera have been enriched with anti-cancer phytometabolites. Some *Ranunculaceae* phytometabolites induce the cell cycle arrest and apoptosis of cancer cells or enhance immune activities, while others inhibit the proliferation, invasion, angiogenesis, and metastasis, or reverse the multi-drug resistance of cancer cells thereby regulating all known hallmarks of cancer. These phytometabolites could exert their anti-cancer activities via multiple signaling pathways. In addition, absorption, distribution, metabolism, and excretion/toxicity properties and structure/activity relationships of some phytometabolites have been revealed assisting in the early drug discovery and development pipelines. However, a comprehensive review of the molecular mechanisms and functions of *Ranunculaceae* anti-cancer phytometabolites is lacking. Here, we summarize the recent progress of the anti-cancer chemo- and pharmacological diversity of *Ranunculaceae* medicinal plants, focusing on the emerging molecular machineries and functions of anti-cancer phytometabolites. Gene expression profiling and relevant omics platforms (*e.g.* genomics, transcriptomics, proteomics, and metabolomics) could reveal differential effects of phytometabolites on the phenotypically heterogeneous cancer cells.

Keywords: Ranunculaceae phytometabolites, Anticancer activity, Molecular mechanism, Genomics.

1. INTRODUCTION

The bioactive natural products (plant secondary metabolites) are well known to possess therapeutic value for the prevention and treatment of various types and stages of cancer [1, 2]. *Ranunculaceae* phytometabolites exhibit promising effects against cancer, many of which modulate signaling pathways that are key to cancer initiation and progression, and enhance the anticancer potential of clinical drugs while reducing their toxic side effects. Although some *Ranunculaceae* phytometabolites were isolated decades ago, this review focuses on pharmacological properties and the latest advances in molecular mechanisms and functions. We discuss our current state of knowledge for adjuvant potential, and anti-cancer activity of *Ranunculaceae* phytometabolites

in vitro and *in vivo*, and highlight their abilities to modulate the hallmarks of cancer. In this article, “anti-cancer” refers to the ability of the compound to inhibit tumor cell proliferation or induce cell death of tumor cells. The indicated activity in cultured cells is usually regarded as the “*in vitro*” result, while the indicated activity in experimental animals, tumor xenografts, or human tumor samples is usually regarded as the “*in vivo*” result. The phytometabolites found to exhibit the cytotoxic or anti-proliferative activities considered to be promising candidates for screening of antitumor phytometabolites.

The *Ranunculaceae* family (eudicot Ranunculales) consists of at least 62 genera and 2 200 species, and 42 genera and about 720 species are distributed throughout Mainland China, most of which are found in the southwest mountainous region [2, 3]. In traditional Chinese medicine (TCM), at least 13 *Ranunculaceae* genera are used in heat-clearing and detoxification (Qing Re Jie Du in TCM), 13 genera used in ulcer disease and sore (Yong Ju Chuang Du in TCM), and seven genera used in swell-reducing and detoxification (Xiao

*Address correspondence to these authors at the Biotechnology Institute, School of Environment and Chemical Engineering, Dalian Jiaotong University, Dalian 116028, China; Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, Beijing 100193, China; Tel: 0086-411-84572552; E-mails: hao@djtu.edu.cn; pexiao@implad.ac.cn

Zhong Jie Du in TCM) [2, 4]. These genera may contain useful phytometabolites that can be used to combat against cancer. Extracts and/or isolated phytometabolites of at least 17 genera have shown anti-cancer/cytotoxic activities toward various tumor cells [1, 2, 5-7]. The distribution of anti-cancer phytometabolites within *Ranunculaceae* is not random but phylogeny-related [8]. For instance, *Ranunculus*, *Clematis*, *Pulsatilla*, *Anemone*, and *Nigella* are rich in pentacyclic triterpene saponins (e.g. Fig. 1, structures 1-6); *Actaea* and *Cimicifuga* are rich in tetracyclic triterpene saponins, diterpenoids, triterpenoids, and monoterpenes (e.g. Fig. 2, structures 7-13), which are also found in *Thalictrum* and *Anemone*; isoquinoline alkaloids (e.g. Fig. 3, structures 14-17) are abundant in *Asteropyrum*, *Caltha*, *Nigella*, *Delphinieae*, *Adonideae*, *Thalictrioideae*, and *Coptidoideae*; diterpenoid alkaloids (e.g. Fig. 3, structures 18-20) are abundant in *Delphinium*, *Consolida*, and *Aconitum*, but are also found in *Nigella* and *Thalictrum*. The same class of phytometabolite, e.g., saponins, could exert the anti-cancer activity via multiple molecular pathways, as schematically depicted below in (Fig. 5), while the same signaling pathway could be the target of distinct phytometabolites, including thymoquinone (TQ, Fig. 2, structure 13), and berberine (Fig. 3, structure 14), as reviewed in [9-13].

2. CELL DEATH PATHWAYS

As cancer cells exhibit multiple mechanisms to resist the induction of programmed cell death (apoptosis), the modulation of apoptotic signaling pathways by *Ranunculaceae* phytometabolites has been shown to constitute a key event in their anticancer activities, as reviewed elsewhere [10, 12, 13]. In addition, cell cycle arrest, autophagy modulation, cell senescence and other pathways are also involved in anti-cancer molecular mechanisms induced by various *Ranunculaceae* phytometabolites, as reviewed in [10, 12, 13].

2.1. Saponins

2.1.1. *Clematis*

Saponins, which are abundant in *Ranunculaceae*, especially in *Clematis*, *Pulsatilla*, *Anemone*, and *Cimicifugeae*, usually exert their anti-cancer activities via induction of cell cycle arrest and apoptosis [1, 2, 6, 7]. The aglycones of *Clematis* pentacyclic triterpene saponins mainly belong to oleanolic type (A), olean-3 β , 28-diol type (B), hederagenin type (C) or hederagenin-11, 13-dien type (D), where types A and C are predominant [1, 7]. Many *Clematis* saponins have cytotoxic activity against human glioblastoma [14], hepatoma [15], cervical cancer [16], leukemia [15, 17], gastric cancer [15, 17], colon cancer [18], and prostate cancer [19]. However, the mechanistic study is scarce. For instance, D-Rhamnose β -hederin (DR β -H, 1 of Fig. 1), an oleanane-type triterpenoid saponin from TCM plant *Clematis ganpiniana*, inhibited phosphoinositide 3-kinase (PI3K)/ protein kinase B (PKB, also known as AKT) pathway and activated an extracellular signal-regulated kinase (ERK) signaling in human breast cancer cells [20]. PI3K inhibitor LY294002 synergistically enhanced DR β -H-induced apoptosis while MEK inhibitor U0126 reduced the apoptosis rate [20]. DR β -H regulated the ratio of pro-apoptotic and anti-apoptotic BCL-2 family proteins. DR β -H induced depolarization of mitochon-

drial membrane potential to release Apoptotic Peptidase Activating Factor 1 (APAF-1) and cytochrome C from the intermembrane space into the cytosol, where they promoted caspase-9 and caspase-3 activation [20].

2.1.2. *Pulsatilla*

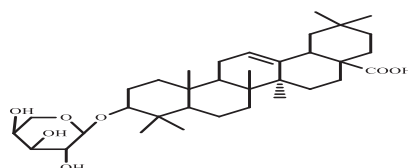
Pulsatilla, *Anemone*, and *Clematis* belong to the tribe Anemoneae, and *Pulsatilla* is evolutionarily more close to *Anemone* than to *Clematis* [2]. Saponins exhibit cytostatic and cytotoxic activity against various cancer cells, but the mechanism is not fully understood. *Pulsatilla koreana* saponin D (SB365) strongly suppressed the growth of hepatocellular carcinoma (HCC) cells in a dose-dependent manner and induced apoptosis by increasing the proportion of sub G1 apoptotic cells from 8% to 21% through induction of BAX expression and caspase-3 cleavage [21]. SB365 effectively suppressed the phosphorylation of PI3K downstream factors, e.g., AKT, mammalian target of rapamycin (mTOR), and p70S6 kinase (p70S6K) serine/threonine kinase *in vitro* and *in vivo* [22, 23]. SB365 suppresses the proliferation of human colon cancer and pancreatic cancer cells and induces apoptosis by modulating the AKT/mTOR signaling pathway [22, 23].

The K_a (absorption rate constant) and P_{app} (apparent permeability coefficient) values of *Pulsatilla* saponin D (Fig. 1, structure 2) are highest in the colon, followed by ileum, jejunum, and duodenum [24]. The *Pulsatilla* saponins were not transported in a concentration dependent manner, and the transporter protein might be involved in their transport. The *Pulsatilla* saponins exhibited rapid absorption, quick elimination, and significant double peak on the plasma concentration-time curve [25]. However, the oral bioavailability is quite low (0.55-2.5%), due to the unfavorable molecular size (≥ 733.5 Da) of pentacyclic triterpene saponins, poor gut absorption, and/or extensive metabolism after absorption [26]. Nanoformulations have to be developed to maximize the anticancer effects of *Pulsatilla* saponins.

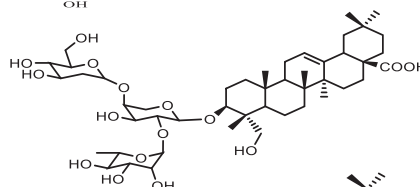
With the aid of the array of the 42 phosphorylated receptor tyrosine kinases (RTKs), SB365 was found to dock at an allosteric site on the epithelial-mesenchymal transition (c-MET) factor and strongly inhibited its expression in gastric cancer cells [27]. The activation of the c-MET signal cascade components, including AKT and mTOR, was inhibited by SB365 in a dose-dependent manner [27, 28]. SB365 inhibited the phosphorylation of c-MET proto-oncogene and the downstream signaling pathway required for growth and survival in the c-MET-amplified HCC827GR non-small cell lung cancer (NSCLC) cells [28]. SB365 suppressed the anchorage-independent growth, migration and invasion and induced apoptosis in HCC827GR cells [28]. *Pulsatilla* saponin A (4 of Fig. 1) of *P. chinensis* may exert its antitumor effect by inducing DNA damage and causing G2/M arrest and apoptosis in multiple cancer cells [29]. *Pulsatilla* saponin A increased p53 and cyclin B protein levels, and decreased BCL-2 protein level [29].

2.1.3. *Anemone*

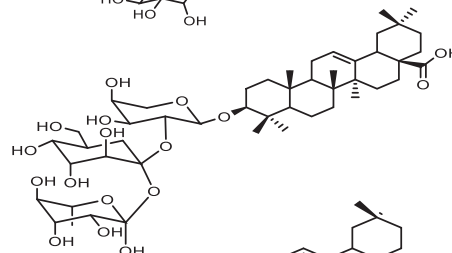
The genus *Anemone*, evolutionarily closely related to *Pulsatilla*, is also rich in therapeutic saponins [2]. Raddeanin A (Fig. 1, structure 3), a pentacyclic triterpene saponin from *Anemone raddeana* (Liang Tou Jian in TCM), inhibits

1) D rhamnose β -hederin (DR β -H)

2) Pulsatilla saponin D



3) Raddeanin A



4) Pulsatilla saponin A

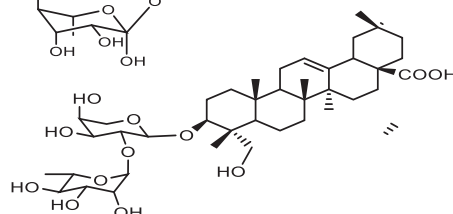
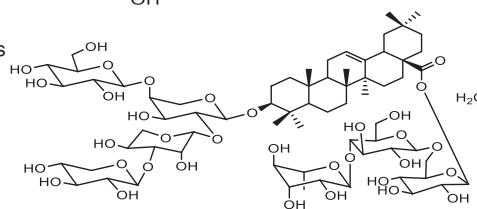
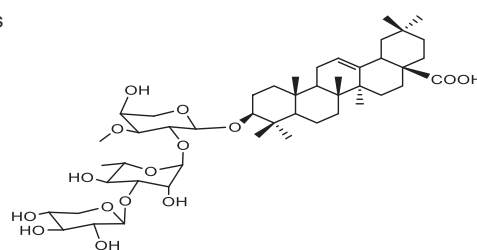
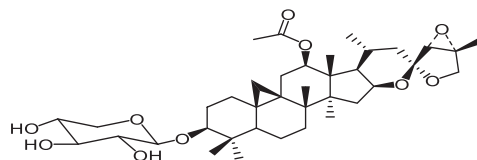
5) saponin B of *Anemone taipaiensis*6) saponin 1 of *Anemone taipaiensis*

Fig. (1). Representative anticancer phytochemicals found in the family Ranunculaceae. Chemical Structures. Pentacyclic triterpene saponins. 1) D rhamnose β -hederin (DR β -H); 2) *Pulsatilla* saponin D; 3) Raddeanin A; 4) *Pulsatilla* saponin A; 5) saponin B of *Anemone taipaiensis*; 6) saponin 1 of *Anemone taipaiensis*.

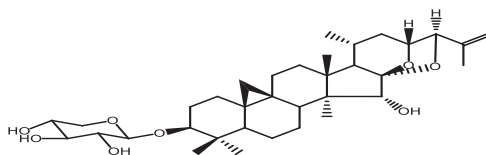
proliferation and induces apoptosis of multiple cancer cells [30, 31]. Raddeanin A increased BAX expression, reduced BCL-2, BCL-xL and survivin protein levels, and significantly activated caspase-3, -8, -9 and poly-ADP ribose polymerase (PARP) [31]. Saponins B (Fig. 1, structure 5), 1 (Fig. 1, structure 6), and 6 of *Anemone taipaiensis* exhibit significant anticancer activity against human leukemia, glioblastoma multiforme (GBM), and HCC [32-34]. Saponin 1 caused characteristic apoptotic morphological changes in GBM cells, which was confirmed by DNA ladder electrophoresis and flow cytometry [32]. Saponin 1 also caused a time-dependent decrease in the expression and nuclear location of nuclear factor-kappa B (NF- κ B) transcription factor, as indicated in [32]. The expression of inhibitors of apoptosis (IAP) family members, e.g., survivin (also known as baculoviral IAP [inhibitor of apoptosis protein] repeat-containing

protein [BIRC5]) and X-linked inhibitor of apoptosis protein (XIAP, also known as BIRC4) was significantly decreased by saponin 1 [32]. Moreover, saponin 1 caused a decrease in the BCL-2/BAX ratio and initiated apoptosis by activating caspase-9 and caspase-3 in the GBM cell lines [32, 33]. Thus, saponin 1 inhibits cell growth of GBM cells at least partially by inducing apoptosis and inhibiting survival signaling mediated by NF- κ B [32, 33]. Saponin B blocked the cell cycle at the S phase [34]. Saponin B induced chromatin condensation of U87MG GBM cells and led to the formation of apoptotic bodies [34]. Data obtained from the Annexin V/propidium iodide assay showed that phosphatidylserine externalization was apparent at higher drug concentrations [34]. Saponin B activated the receptor-mediated pathway of apoptosis via the activation of FAS ligand (FASL), as indicated in [34]. Saponin B increased the BAX and caspase-3

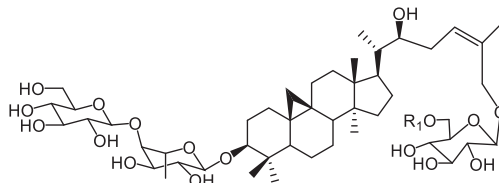
7) Actein



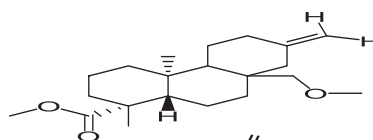
8) Cimiciside E



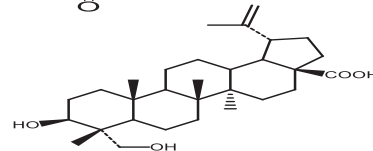
9) 3-O-β-D-glucopyranosyl-(1-4)-β-D-fucopyranosyl (22S, 24Z)-cycloart-24-en-3β, 22, 26-triol 26-O-β-D-glucopyranoside, R1=H



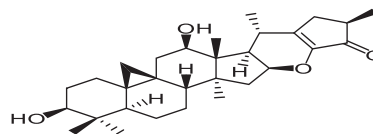
10) dihydroxy-isosteviol methyl ester (DIME)



11) 23-hydroxybetulinic acid (HBA)



12) Cimyunin A



13) Thymoquinone (TQ)

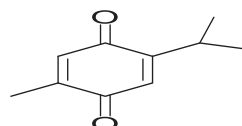


Fig. (2). Representative anticancer phytochemicals found in the family Ranunculaceae. Chemical Structures. Tetracyclic triterpene saponin (7-9). Diterpenoid (10). Triterpenoid (11, 12). Monoterpene (13).

protein levels and decreased the protein expression of BCL-2 [34]. Triterpenoid saponins of *Anemone flaccida* induce apoptosis in human HepG2 hepatoma BEL-7402 cells, and lipopolysaccharide-stimulated human cervical cancer HeLa cells via cyclooxygenase-2 (COX-2, also known as prostaglandin-endoperoxide synthase 2)/prostaglandin E2 (PGE2) pathway [35].

2.1.4. Cimicifugeae

Cycloartane triterpenoids and their saponins are mainly distributed in *Astragalus* (Leguminosae), *Cimicifugeae* and *Thalictrum* (Ranunculaceae), and possess various bioactivities [36]. *Actaea* and *Cimicifuga* are closely related genera of the tribe *Cimicifugeae*, which are closer to *Souliea* than to *Eranthis* [1, 2, 6]. *Beesia* is closer to *Anemonopsis* than to other four genera [1, 2, 6].

Our research group found that 9, 19-cycloartane triterpene glycosides of *Actaea asiatica* had notable cytotoxicity against human hepatocellular carcinoma HepG2 cells and breast cancer MCF-7 cells [37]. The tetracyclic triterpene

saponins and their aglycones displayed the cytotoxic activity against HCC, lung adenocarcinoma [38], gastric cancer [39], leukemia, colon cancer, and breast cancer [40]. Cycloartane glycosides from the roots of *Cimicifuga foetida* (Sheng Ma in TCM) showed significant WNT signaling pathway inhibitory activity, with IC_{50} of 3.33 and 13.34 μ M, respectively [41]. Our research group found that 23-O-acetylcimigenol-3-O-β-D-xylopyranoside could inhibit the proliferation of HepG2 cells with IC_{50} 16 μ M, and could induce apoptosis and G2/M cell cycle arrest [42]. This tetracyclic triterpene saponin is able to cleave PARP, regulate protein expression of BCL-2 family and decrease the expression of cell division cycle protein 2 homolog (CDC2, also known as cyclin-dependent kinase 1, CDK1) and cyclin B (CCNB). 25-Acetyl-7, 8-didehydrocimigenol 3-O-β-D-xylopyranoside is more potent than the parent compound 7, 8-didehydrocimigenol 3-O-β-D-xylopyranoside in inhibiting ER-/HER2 overexpressing human breast cancer MDA-MB-453 cells [43]. HER2 is a receptor tyrosine-protein kinase ERB-2 (also known as CD340 [cluster of differentiation

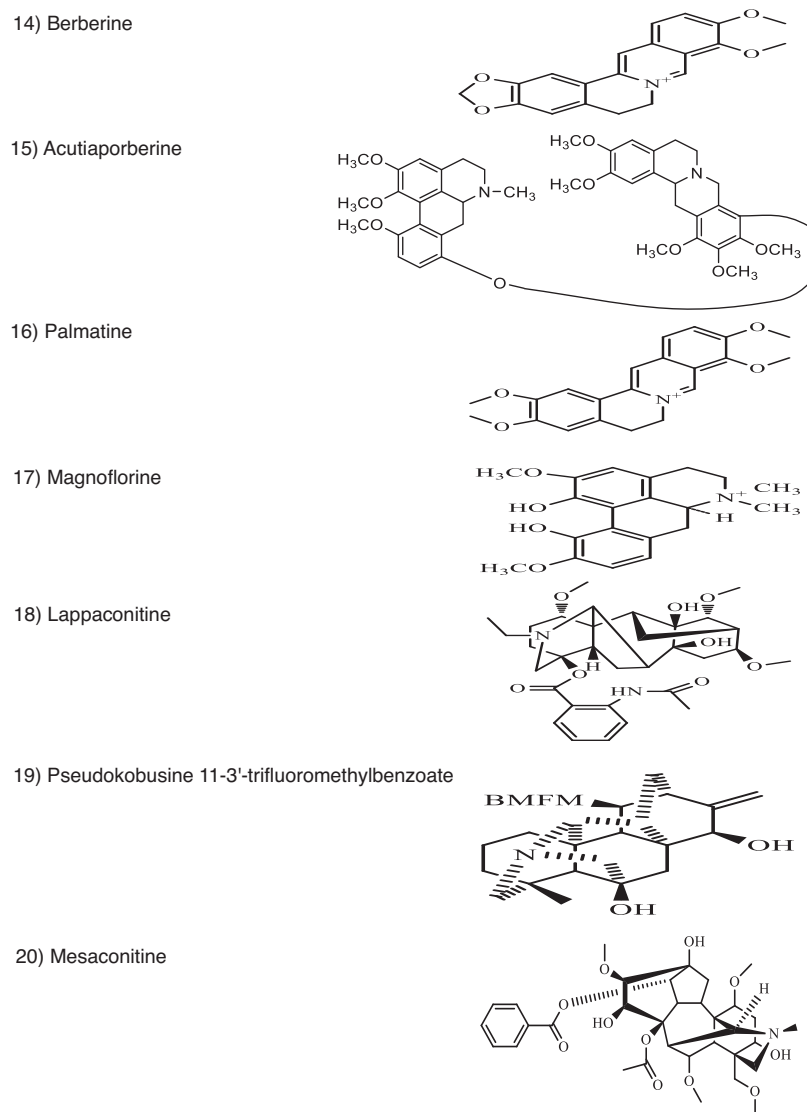


Fig. (3). Representative anticancer phytometabolites found in the family Ranunculaceae. Chemical Structures. Isoquinoline alkaloid (14-17). Diterpenoid alkaloid (18-20).

340], proto-oncogene NEU, or ERBB2), and a member of the human epidermal growth factor (EGF) receptor (EGFR) family [44-48]. Amplification or overexpression of this oncogene has been shown to play an important role in the development and progression of certain aggressive types of breast cancer [44-48].

Cell cycle arrest in human gastric cancer cells was induced by cimicide E (Fig. 2, structure 8) of *C. heracleifolia* (a source plant of Sheng Ma) in S phase at a lower concentration (30 μ M) and G2/M phase at higher concentrations (60 and 90 μ M) [39]. Cimicide E mediated apoptosis through the induction of the caspase cascade for both the extrinsic and intrinsic pathways. Our research group found that 25-anhydrocimigenol-3-O- β -D-xylopyranoside of *Souliea vaginata* (Huang San Qi in TCM) caused apoptosis and G0/G1 cell cycle arrest in HepG2 cells, and exhibited a dose-dependent inhibition of tumor growth on mice implanted with H22 *in vivo* [43].

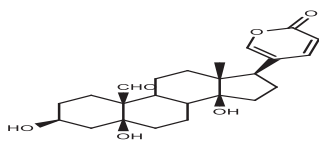
Actein (Fig. 2, structure 7), a triterpene glycoside from black cohosh (*Actaea racemosa*, *Cimicifuga racemosa*),

strongly inhibited the growth of human breast cancer cells and induced a dose-dependent release of calcium from endoplasmic reticulum (ER) and mitochondria into the cytoplasm [49]. Heparin, the antagonist for the ER inositol 1,4,5-trisphosphate receptor (IP3), blocked this release [49]. Heparin partially blocked the growth inhibitory effect of actein, while the MEK inhibitor U0126 enhanced it [49]. Actein synergized with the ER mobilizer thapsigargin and preferentially inhibited the growth of human embryonic kidney 293T cells [49]. Nanoparticle liposomes increased the growth inhibitory activity of actein [49]. Actein alters the activity of the ER IP3 receptor and Na, K-ATP-ase, induces calcium release, modulates the NF- κ B and mitogen-activated protein kinase (also known as MAP2K, MEK, MAPKK) pathways, and causes cytochrome C release from mitochondria, which may partially explain the anti-cancer effect of actein [49].

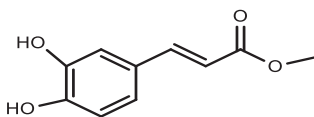
Extracts (enriched for triterpene glycosides) and phytometabolites from the North American perennial black cohosh (*Actaea racemosa* L; *Ranunculaceae*) and related *Cimi-*

cifuga species were shown to inhibit proliferation of human breast cancer cells (MDA-MB-453 cells and MCF7 [ER+/Her2 low] cells), as indicated in [50]. Forced expression of exogenous HER2 in human breast cancer MCF7 (ER+/Her2 low) cells made these cells more sensitive than the parental MCF7 cells to the growth inhibitory effects of actein, indicating that HER2 protein plays a role in the actein ability to inhibit tumor cell proliferation [50]. Actein was also found to alter the distribution of actin filaments and induced apoptosis in these cells, suggesting that actein might have both cytostatic and cytotoxic activity [50].

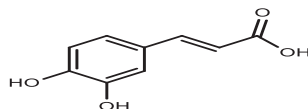
21) Hellebrigenin



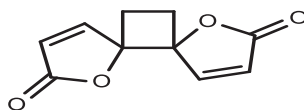
22) Methyl caffeate



23) Caffeic acid



24) Anemonin



25) Ranuncululin

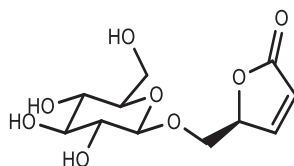


Fig. (4). Representative anticancer phytochemicals found in the family Ranunculaceae. Chemical Structures. Cardioactive steroid (21). Phenolic acid (22-25). Lactone (24, 25).

3-O- β -D-glucopyranosyl-(1 \rightarrow 4)- β -D-fucopyranosyl (22S, 24Z)-cycloart-24-en-3 β , 22, 26-triol 26-O- β -D-glucopyranoside (Fig. 2, structure 9), isolated from *Thalictrum fortunei*, caused apoptosis and mitochondrial membrane potential loss in human hepatoma Bel-7402 cells [46]. The intracellular reactive oxygen species (ROS) were markedly upregulated by compound 1, as indicated in [51]. Compound 1 significantly increased the expression levels of cleaved caspase-3, p53 and BAX protein, and decreased the expression of BCL-2 protein [51].

2.2. Terpenoid

Dihydroxy-isosteviol methyl ester (DIME, Fig. 2, structure 10), a diterpene isolated from *Pulsatilla nigricans*, caused a significant decrease in cell viability, induced nuclear condensation and inter-nucleosomal DNA fragmentation [52]. DIME interacted with calf thymus DNA, bringing apparent changes in structure and conformation [52]. 23-

Hydroxy-betulinic acid (Fig. 2, structure 11) of *Pulsatilla chinensis* could promote cell cycle arrest in S phase and induce apoptosis of human chronic myelogenous leukemia K562 cells *via* intrinsic pathway [53]. HBA disrupts mitochondrial membrane potential significantly and selectively downregulates the levels of BCL-2, survivin and upregulates BAX, cytochrome C, cleaved caspase-9 and -3 [53].

Thymoquinone (TQ, 2-methyl-5-isopropyl-1, 4-benzoquinone, Fig. 2, structure 13), a monoterpene abundant in *Nigella sativa*, down-regulates E2F-1 protein and androgen receptors, and causes apoptosis in cancer cells [54]. Thymoquinone downregulates the expression of BCL-2 and other anti-apoptotic proteins, and upregulates the expression of pro-apoptotic proteins (caspase-3, -8, -9 & BAX) expression in cancer cells, as indicated in [55, 56]. Thymoquinone induces apoptosis in human colon cancer HCT116 cells through inactivation of STAT3 by blocking JAK2- and SRC-mediated phosphorylation of EGFR tyrosine kinase [10]. Recently, a serine/threonine-protein kinase PAK1 was identified as a novel target for thymoquinone [57]. Thymoquinone-induced conformational changes in PAK1 protein interrupt prosurvival MEK-ERK signaling in human colorectal cancer and enhance apoptosis [57]. Thymoquinone targets various components of intracellular signaling pathways, particularly upstream kinases and transcription factors, which are aberrantly activated during carcinogenesis [11]. The above mechanisms exemplify how thymoquinone causes apoptosis in cancer cells.

Thymoquinone also displays anti-proliferative and cytostatic effects [58]. For instance, thymoquinone inhibits NF- κ B activation induced by various carcinogens, and controls oncogenic expression [58]. In N-nitrosodiethylamine-induced rat HCC, thymoquinone strongly induced G1/S arrest in cell cycle transition [59]. Thymoquinone suppresses the activation of AKT and ERK, and activates c-Jun NH₂-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) pathways, as described in [60, 61]. Thymoquinone pretreatment overcomes the pancreatic cancer cell resistance potentiating the anticancer effect of gemcitabine through abrogation of NOTCH1, and modulation of PI3K/AKT/mTOR signaling pathways in pancreatic cancer [62]. Thymoquinone inhibited topoisomerase II α activity when incubated with the enzyme prior to the addition of DNA, but enhanced enzyme-mediated DNA cleavage ~5-fold, which is similar to the increase seen with the anticancer drug etoposide [63]. Thymoquinone inhibits thymidine incorporation during DNA synthesis, and inhibits cancer cell growth [63, 64].

The polo-box domain (PBD), a unique functional domain of polo-like kinase (PLK), is being targeted to develop PLK1-specific inhibitors [65]. Thymoquinone and its derivative Poloxin are known PBD inhibitors and were shown to increase cell population in S phase and in G2/M, in a p53-independent manner [65]. Poloxin and Thymoquinone did not increase population of cells staining positively for phosphorylated (p)-histone H3 and M-phase phosphoprotein-2 (MPM2, also known as MPHOSPH, the protein involved in The transition of G2 to M phase), as indicated in [65]. However, both PBD inhibitors caused an increase in p21^{WAF1} and cell cycle arrest at the S-phase, indicating that they affected interphase before mitotic entry [65].

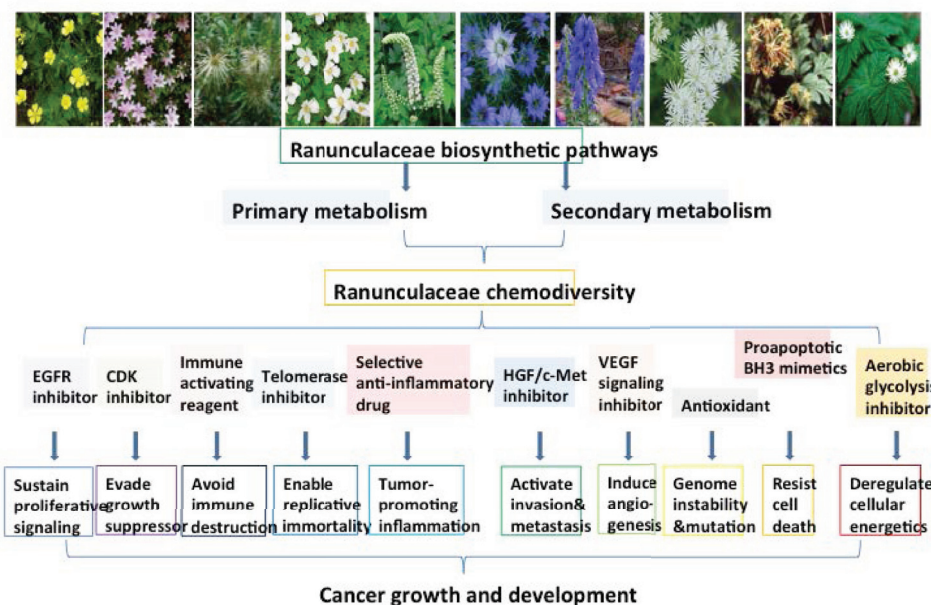


Fig. (5). The cancer hallmarks modulated by Ranunculaceae phytometabolites. Ranunculaceae phytometabolites regulate all hallmarks of cancer, as defined by Hanahan and Weinberg [134]. The putative PARP inhibitor has not been identified from Ranunculaceae plants. Instead, various antioxidants found in Ranunculaceae might play important roles in fighting against genome instability and mutation of cancer cells.

Cell death may occur in several mechanisms, including apoptosis, necrosis (see below, anti-inflammatory activity), and autophagy. Autophagy is a catabolic process that maintains cellular homeostasis in response to various cellular stress factors. Thymoquinone-treated head and neck squamous cell carcinoma cells showed increased levels of autophagic vacuoles and specific autophagy markers LC3-II proteins [66]. Thymoquinone treatment also increased the accumulation of autophagosomes [66]. A BALB/c nude mouse xenograft model showed that thymoquinone administered by oral gavage reduced tumor growth via induced autophagy and apoptosis *in vivo* [66]. Bafilomycin A1, an autophagy inhibitor, enhanced thymoquinone cytotoxicity but did not promote apoptosis [66]. Cell viability was eradicated in autophagy-defective cells [66]. These results imply that inhibition of autophagy is an emerging strategy in cancer therapy [66]. In human colon cancer CPT-11-R LoVo cells, thymoquinone caused mitochondrial outer membrane permeability and activated autophagic cell death [67]. JNK and p38 inhibitors (SP600125 and SB203580, respectively) reversed TQ autophagy [67]. Thymoquinone activated apoptosis before autophagy, and the direction of cell death was switched toward autophagy at initiation of autophagosome formation [67].

GBM cells may be dependent on the autophagic pathway for survival [68]. Thymoquinone inhibits autophagy and induces cathepsin-mediated, caspase-independent cell death in GBM cells [68]. Thymoquinone induces lysosome membrane permeabilization, which results in a leakage of cathepsin B into the cytosol and mediates caspase-independent cell death that can be prevented by pre-treatment with a cathepsin B inhibitor [68]. Thymoquinone induced apoptosis appears to be caspase-independent due to failure of the caspase inhibitor z-VAD-FMK to prevent cell death and absence of the typical apoptosis signature DNA fragmentation [68].

2.3. Alkaloid

Thalictrum, *Coptis*, and *Hydrastis* are rich in isoquinoline alkaloids [2]. The anticancer effects of *Coptis Chinensis* (Huang Lian in TCM) can be ascribed to its TCM trait of removing damp-heat, fire and toxicity [69]. Berberine, a major isoquinoline alkaloid of Huang Lian and other related species (Fig. 3, structure 14), is accumulated inside prostate cancer cells that were in G1 phase of the cell cycle and enhanced apoptosis [70]. Berberine inhibited the expression of prostate-specific antigen, and attenuated EGFR activation following EGF treatment *in vitro* [70]. Berberine induces apoptosis via the mitochondrial pathway in liver cancer cells [71]. Berberine increased the expression of BAX, followed by the activation of the caspase cascade. Pro-caspase-9 and its effectors, pro-caspase-3 and -7, were activated by berberine. Berberine activates the transcription factor Egr-1 and consequently induces the expression of non-steroidal anti-inflammatory drug (NSAID)-activated gene (NAG-1), which mediates the drug-induced pro-apoptotic action in HCC HepG2 cells [72]. Other protoberberine-type alkaloids in Huang Lian might exhibit synergistic effects, while showing the anti-cancer effects [69, 70].

Berberine significantly induced the mRNA expression of Forkhead box family members, (FOX)-O1 and O3A [73]. Their phosphorylation-mediated cytoplasmic sequestration followed by degradation was prevented by berberine-induced downmodulation of the PI3K/AKT/mTOR pathway, which promoted a nuclear retention of FOXO proteins [73]. Phosphatase and tensin homolog (PTEN), a tumor suppressor protein and negative regulator of the PI3K/AKT axis, was upregulated while phosphorylation of its Ser380 residue (possible mechanism of PTEN degradation) was significantly decreased in berberine-treated HCC HepG2 cells [73]. Berberine induced a significant increase in transcriptional

activity of FoxO, which effectively enhanced BH3-only protein BIM expression, followed by the direct activation of pro-apoptotic protein BAX, increased BAX/BCL-2 ratio, mitochondrial dysfunction, caspases activation, and DNA fragmentation [73]. Silencing of BIM expression partially restored HCC HepG2 cell viability during berberine exposure, implying the pivotal role of BIM in berberine-mediated cytotoxicity [73]. Acutiaporberine (Fig. 3, structure 15), a bis-alkaloid isolated from *Thalictrum acutifolium*, may be a natural potential apoptosis-inducing agent for highly metastatic lung cancer [74]. Palmatine (16 of Fig. 3, structure 16), but not berberine, is a cell cycle blocker in G1 of A549 adenocarcinoma cells [75]. Berberine induced cell cycle arrest at the G2/M phase in human promyelocytic leukemia HL-60 cells and murine myelomonocytic leukemia WEHI-3 cells, which was accompanied by increased levels of WEE1 and 14-3-3 σ , and decreased levels of CDC25C, CDK1, and cyclin B1 (CCNB1), while CDK2 expression was not affected by berberine [76].

In melanoma cell lines, berberine at low doses (12.5-50 μ M) is concentrated in mitochondria and promotes cell cycle arrest at the G1 phase [77]. Higher doses (> 50 μ M) result in cytoplasmic and nuclear accumulation of berberine, and G2 phase arrest [77]. DNA synthesis is not markedly affected by low doses of berberine, but 100 μ M is strongly inhibitory [77]. Notably, 100 μ M of berberine inhibits cell growth with relatively little induction of apoptosis [77]. Berberine displays multiphasic effects in malignant cell lines, which are correlated with its concentration and intracellular distribution [13, 78]. These results help explain some of the conflicting information regarding the effects of berberine, which may be more as a cytostatic agent than a cytotoxic compound [13, 78].

Berberine may inhibit protein synthesis, histone deacetylase (HDAC), or AKT/mTOR pathways [78]. Berberine induced endoplasmic reticulum (ER) stress and autophagy, which was associated with activation of AMP-activated protein kinase (AMPK), as reviewed in [13, 78]. However, berberine did not alter mTOR or HDAC activities in MDA-MB-231 human breast cancer cells [78]. Berberine induced the acetylation of α -tubulin, a substrate of HDAC6 [78]. In addition, the combination of berberine and suberoylanilide hydroxamine (SAHA), a pan-HDAC inhibitor, synergistically inhibited cell proliferation and induced cell cycle arrest. Berberine induced both autophagy and apoptosis in HCC cells, and the expression of basigin (also known as extracellular matrix metalloproteinase inducer [EMMPRIN] or cluster of differentiation 147 [CD147]) was downregulated by berberine [79].

Berberine primarily exerts its anti-cancer effect by inducing cell cycle arrest, apoptosis, and autophagy [13]. However, berberine inhibited GBM cells through induction of cellular senescence [80]. Berberine drastically reduced the level of EGFR, and the downstream c-RAF-MEK-ERK signaling pathway was remarkably inhibited, whereas AKT phosphorylation was not altered in GBM cells [80]. Pharmacologic inhibition or RNA interference of EGFR similarly induced cellular senescence of GBM cells, which was rescued by introduction of a constitutive active mitogen-activated protein kinase kinase [80]. Berberine potently in-

hibited the growth of tumor xenografts, which was accompanied by downregulation of EGFR and induction of senescence [80].

Diterpenoid alkaloids are abundant in *Aconitum* and *Delphinium*, and are known to exhibit anti-cancer activities, as reviewed in [1, 2, 5]. For instance, lappaconitine (Fig. 3, structure 18) caused G0/G1 cell cycle arrest, apoptosis and downregulation of cyclin E1 (CCNE) gene expression of non-small cell lung cancer [81]. Taipenin A, a C19-diterpenoid alkaloid from the roots of *Aconitum taipaicum*, upregulated the protein expression of BAX and caspase-3 and downregulated the expression of BCL-2 and cyclin D1 (CCND1) [82]. The *Delphinium* diterpenoid alkaloids were found to be cytotoxic against human adenocarcinoma A549 cells with the IC₅₀ values ranged from 12.03 to 52.79 μ M, as indicated in [83].

2.4. Cardioactive Steroid

Hellebrigenin (Fig. 4, structure 21) of *Helleborus*, one of bufadienolides belonging to cardioactive steroids, potently reduced the viability and colony formation of human HCC cells [84]. Hellebrigenin triggered DNA damage through DNA double-strand breaks and subsequently induced cell cycle G2/M arrest associated with up-regulation of phosphorylated (p)-ataxia telangiectasia mutated (ATM) kinase, p-checkpoint kinase (CHK)-2, p-CDK1 and cyclin B1 (CCNB1), and down-regulation of p-CDC25C, as described in [84]. Hellebrigenin induced mitochondrial apoptosis, characterized by BAX translocation to mitochondria, disruption of mitochondrial membrane potential, release of cytochrome C into cytosol, and sequential activation of caspases and PARP, as indicated in [84]. AKT expression and phosphorylation were inhibited by hellebrigenin, whereas AKT silencing with siRNA abolished cell cycle arrest but enhanced apoptosis induced by hellebrigenin [84]. Activation of AKT by human insulin-like growth factor (IGF)-1 could obviously attenuate hellebrigenin-induced cell death [84].

2.5. Plant Extract

The plant extracts are traditionally obtained from the whole plant, root, rhizome, stem, leaf, flower, and other uncultured/cultured tissues/cells, which may contain multiple therapeutic phytochemicals. For instance, *Pulsatilla chinensis* (Bai Tou Weng in TCM), rich in pentacyclic triterpene saponins, is among the top 10 potent anti-mitotic inhibitors (independent of toxicity) in a recent study that screens 897 aqueous extracts of commonly used natural products (0.00015- 0.5 mg/mL) relative to paclitaxel for anti-mitotic effects on human breast cancer MDA-MB-231 cells [85]. *Pulsatilla koreana* extract strongly suppressed the growth of HCC and anaplastic thyroid cancer cells in a dose-dependent manner [86, 87]. Apoptosis induced by *Pulsatilla koreana* extract was observed using DAPI and TUNEL staining assays and the cleaved PARP and caspase-3 were increased in HCC Huh-7 cells [86].

In MDA-MB-453 human breast cancer cells, a methanolic extract of *Cimicifuga racemosa* caused a significant increase in expression of ER stress (e.g., GRP78), apoptotic (GDF15), lipid biosynthetic (INSIG1 and HSD17B7) and phase I (CYP1A1) genes, and decrease in expression of cell

cycle regulators, such as HELLS and PLK4, as described elsewhere [88]. A lipophilic *C. racemosa* rhizome extract significantly regulated 431 genes in human breast cancer MCF-7 cells, many of which are involved in anti-proliferation and pro-apoptotic networks [9]. The expression pattern differed from those induced by 17 β -estradiol or the estrogen receptor antagonist tamoxifen [88]. Our group found that the ethyl acetate fraction (EAF) of *C. foetida* aerial part induced G0/G1 cell cycle arrest at lower concentration (25 μ g/mL) in human HCC HepG2 cells, and triggered G2/M arrest and apoptosis at higher concentrations (50 and 100 μ g/mL) [89]. An increase in the ratio of BAX/BCL-2, activation of downstream effector caspase 3, and cleavage of PARP were implicated in EAF-induced apoptosis [89]. EAF inhibited the growth of the implanted mouse H22 tumor in a dose-dependent manner with the growth inhibitory rate of 63.32% at 200 mg/kg, as described in [89]. Total triterpenoid glycosides of *C. dahurica* (a source plant of Sheng Ma) aerial part showed the similar effects as EAF [90]. An isopropanolic extract of *C. racemosa* induced apoptosis of human prostate androgen-dependent and -independent carcinoma cells, accompanied by the increased degradation of cytokeratin 18 (a known caspase substrate) [91].

The extract from *Coptis chinensis* has a wide effect on cell signaling, including cell cycle regulation (CDK6, CDK4, cyclin B1, cyclin E, cyclin D1, p27), cell adhesion (E-cadherin, osteopontin), differentiation, apoptosis (p-STAT3, p53, BRCA1), cytoskeleton (p-PKC α/β II, Vimentin, p-PKC α), MAPK signaling (RAF-1, p-p38 MAPK, p-ERK1/2), and the PI3K signaling pathway (p-AKT, p-PTEN), as described in [92]. The phytochemicals from *C. chinensis* are suggested to be novel therapeutic drugs for squamous cell carcinoma [92].

3. MICRORNAs, DNA DAMAGE, EPIGENETIC REGULATION

3.1. MICRORNAs

MicroRNAs (miRs) are small 18-24-nucleotide non-coding RNAs, which repress target gene expression largely by modulating translation and mRNA stability [93]. MicroRNA expression is deregulated in many types of cancer [93]. Drug-induced microRNAs have emerged as key regulators in guiding their pharmacological effects [93, 94]. For instance, PEG4000-TQ-nanoparticles could expressively increase the expression of miR-34a through p53 [94]. MiR-34a up-regulation directly downregulated RAC1 expression followed by actin depolymerization and disrupted the actin cytoskeleton, which leads to significant reduction in the lamellipodia and filopodia formation on cell surfaces [94]. PEG4000-TQ-nanoparticles circumvent TQ's poor aqueous solubility, thermal and light volatility, and consequently minimal systemic bioavailability, and thus might be more powerful in retarding cancer cell migration [94].

A berberine-induced miR-21-3p directly down-regulates methionine adenosyltransferases 2A and 2B inhibiting the growth of human HCC Hep2B cells [95]. Overexpression of miR-21-3p increased intracellular S-adenosylmethionine content, which is disadvantageous for HCC cell growth [95]. In NVP-AUY922 (second generation Hsp90 inhibitor)-insensitive colorectal cancer cells, the combination of NVP-

AUY922 and berberine caused cell growth arrest through inhibiting CDK4 expression and induction of miR-296-5p-mediated suppression of Pin1- β -catenin-cyclin D1 signaling pathway [96]. The miR-93 levels in cisplatin-resistant cells were higher than that in cisplatin-sensitive cells, as reviewed elsewhere [93, 97]. Berberine could inhibit miR-93 expression and increase its target tumor suppressor PTEN [97]. Berberine modulated the sensitivity of cisplatin through miR-93/PTEN/AKT signaling pathway in the ovarian cancer cells [97]. In multiple myeloma cells, berberine significantly down-regulated miRNA clusters miR-99a, miR-125b, miR-17-92, and miR-106 -25 [98]. RAC1, NF- κ B, c-MYC, c-JUN, and CCND1, the top five differentially regulated genes, might play key roles in the progression of multiple myeloma [98]. Three common signaling pathways (TP53, ERB, and MAPK) link the three miRNA clusters and the five key mRNAs [98].

3.2. DNA Damage and Epigenetic Regulation

3.2.1. Thymoquinone

Human telomere DNA regulates gene transcriptions and folds up into G-quadruplex structures that inhibit telomerase over-expression in cancer cells, as reviewed in [99]. Thymoquinone interacts with G-quadruplex on two binding sites adjacent to the TTA sequence loop [99]. Thymoquinone is preferentially binding to G-quadruplex over duplex, which is explained by an intercalation-binding mode based on π - π stacking [99]. Thymoquinone might act as a G-quadruplex DNA stabilizer and subsequently inhibit telomerase and cancer proliferation [99].

Thymoquinone induced DNA damage, cell cycle arrest and apoptosis in GBM cells [100]. Thymoquinone facilitated telomere attrition by inhibiting the activity of telomerase. DNA-dependent protein kinase (DNA-PK) is a nuclear, serine/threonine protein kinase consisting of a 470-kDa catalytic subunit of DNA-PK and a heterodimeric regulatory complex KU70/80, as reviewed elsewhere [100]. This enzyme is essential for the repair of DNA double-strand breaks and mediates DNA repair *via* phosphorylation of downstream DNA binding proteins such as p53, as reviewed in [100]. Telomeres in GBM cells with DNA-PK were more sensitive to thymoquinone-mediated effects as compared to those cells deficient in *PRKDC/DNA-PK* gene [100].

Plant-derived antioxidants can switch to prooxidants even at low concentrations in the presence of transition metal ions such as copper, as reviewed in [101]. It is noted that tissue, cellular and serum copper levels are considerably elevated in various malignancies [101]. Thymoquinone is able to cause an oxidative cellular DNA breakage, which can be inhibited by copper-chelating agents and scavengers of ROS [101]. Thymoquinone targets cellular copper in human prostate cancer cells (PC3 and LNCaP) causing a prooxidant cell death [101]. Such a prooxidant cytotoxic mechanism could explain the anticancer activity of plant-derived antioxidants [101]. The *in silico* target identification analysis suggests that HDAC2 proteins could act as the potential targets of thymoquinone [102]. In cancer cells, thymoquinone treatment resulted in a significant HDAC2 inhibition and histone hyperacetylation, providing the first evidence of thymoquinone as a potential epigenetic modulator [103].

3.2.2. Berberine and Other Alkaloids

Berberine was shown to induce DNA repair in a panel of DNA repair-deficient chicken B lymphocyte (DT40) clones, as described in [104]. For example, DT40 cells (*Rev3*^{-/-}), deficient in Reversionless-3 (*Rev3*) gene, were found to be hypersensitive to berberine, as described in [104]. *Rev3* protein (also known as DNA polymerase zeta catalytic subunit) is an enzyme that interacts with *Rev7* to form Pol ζ , a B family polymerase [105]. Since, Pol ζ lacks 3' to 5' exonuclease activity, it requires *Rev3* protein to contribute into translesion synthesis, a DNA damage tolerance process allowing the DNA replication machinery to replicate past DNA lesions [105]. Following berberine treatment, cell cycle analysis identified that G2/M arrest was increased in *Rev3*^{-/-} cells [104]. Furthermore, berberine also induced a significant increase in double-strand breaks in *Rev3*^{-/-} cells compared to wild-type cells, as revealed by chromosomal aberration analysis [104]. These results suggest that berberine is able to induce DNA damage, and that the *Rev3*-associated DNA repair pathway participates in the DNA repair process [104].

Histone H2AX is phosphorylated at the position S139 (also known as γ H2AX) upon DNA damage, thereby its foci formation recognized by specific antibodies could be often used to detect DNA damage in many cells subjected to stress. For example, berberine was found to induce significant concentration- and time-dependent increases in DNA double-strand breaks in human osteosarcoma MG-63 cells, confirmed by the immunodetection of γ H2AX foci, as indicated in [106]. Berberine and palmatine appeared to be the most potent DNA damage inducers in HCC HepG2 cells [107]. Berberine and palmatine suppressed the activities of both topoisomerase (TOP)-1, and II as described in [107]. In berberine-treated cells, DNA damage was shown to be directly associated with the inhibitory effect of TOP2, but not TOP1. DNA damage was also observed in cells treated with *Hydrastis* (also known as golden seal) extracts and the extents of DNA damage were positively correlated with the berberine contents [107]. The TOP2 inhibitory effect may contribute to berberine- and golden seal-induced genotoxicity [107].

Berberine increased the radio-sensitivity of the human breast cancer MCF-7 cells and MDA-MB-468 cells [108]. The radiation-induced G2/M cell cycle delay was reduced in MCF-7 cells pre-treated with berberine, as berberine caused cell cycle arrest at the G1/S phase [108]. Berberine pre-treatment prolonged the persistence of DNA double-stranded breaks in the human breast cancer MCF-7 cells [108]. The protein levels of the DNA repair protein Rad51 homolog 1 (RAD51) were decreased by berberine, and in the cells pre-treated with 15 μ M berberine for 24 h, RAD51 protein decreased significantly at 0, 2, 6 and 24 h after X-ray exposure [108]. Berberine sensitizes human breast cancer cells to ionizing radiation by inducing cell cycle arrest and the down-regulation of the homologous recombination repair protein, RAD51, as indicated in [108]. This emerging evidence shows that berberine may be a promising radio-sensitizer for the treatment of breast cancer.

Palmatine and berberine can induce the formation of G-quadruplex, as well as increase its stability [109]. The un-

saturated ring C, N⁺ positively charged centers, and conjugated aromatic rings are key factors to increase the stabilization ability of palmatine and berberine [109]. Interactions between berberine and the pBR322 plasmid DNA suggest biologically significant DNA-binding abilities of berberine [109]. The possible binding sites of berberine on histone proteins were determined by molecular docking [110]. In human cervical cancer HeLa cells, berberine might modulate p53 and viral oncoproteins HPV-18 E6-E7 via epigenetic modifications [110]. Berberine can repress the expression of DNA methyltransferase (DNMT)-1 and DNMT3B, which triggers hypomethylation of tumor protein (TP)-p53 by changing the DNA methylation level in human multiple melanoma cell U266 [111].

3.2.3. Phenolic Acid

Among *C. racemosa* phenylpropanoids tested for protecting against menadione-induced DNA damage, methyl caffeate (Fig. 4, structure 22) is most potent, followed by caffeic acid (Fig. 4, structure 23), ferulic acid, cimiracemate A, cimiracemate B, and fukinolic acid, all of which has antioxidant activity [112]. The methanol extracts of *C. racemosa* showed dose-dependent decreases in DNA single-strand breaks and oxidized bases induced by the quinone menadione.

4. OXIDATIVE PROCESS AND METABOLISM

4.1. Antioxidant vs. Prooxidant

Plant-derived dietary antioxidants have attracted considerable interest in recent decades for their chemopreventive and cancer therapeutic abilities. *Pulsatilla chinensis* polysaccharides enhance superoxide dismutase and catalase enzyme activities and lower MDA levels in the plasma of glioma bearing mice [113]. Polysaccharides could relieve the liver and kidney damage of glioma bearing mice, and decrease plasma levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and urea [113].

α -Hederin, a pentacyclic triterpene saponin abundant in *Nigella sativa* and *Clematis*, increases the production of ROS in cancer cells, altering mitochondrial functions and causing apoptosis [114, 115]. Inducers of phase II enzyme play an important role in cancer chemoprevention [116]. Quinone reductase (QR), a typical phase II enzyme, can convert toxic quinones to hydroquinones and reduce oxidative cycling. The oleanane saponins of *P. chinensis* exhibited more potent quinone reductase inducing activities than the lupine saponins, and the CD value (concentration required to double the quinone reductase-inducing activity of the control) of the compound with the most potent quinone reductase-inducing activity was 1.1 μ M, as indicated in [117].

Thymoquinone inhibits carcinogen-metabolizing enzyme activity and oxidative damage of cellular macromolecules, and attenuates inflammation [11]. The anti-proliferative and pro-apoptotic effects of thymoquinone in breast cancer are mediated through p38 MAPK phosphorylation via ROS generation [118]. *N. sativa* extract increases the activities of antioxidant enzymes, such as superoxide dismutase, chloramphenicol transferase, and glutathione peroxidase, and protects cells against cancer [119, 120].

Berberine induced ROS production for up to six hours of incubation with human gastric cancer SNU-5 cells [121]. In PANC-1 and MIA-PaCa2 pancreatic cancer cells, apoptosis was induced by berberine via the production of ROS rather than caspase-3 and -7 activation [122]. In HepG2 cells, berberine treatment led to a pronounced increase in JNK phosphorylation and enhanced ROS generation, lipid peroxidation, decreased activities of superoxide dismutase (SOD) and catalase (CAT), and diminished glutathione levels, as reviewed in [13, 71, 73]. Berberine and d-limonene (a monoterpene) at a ratio of 1:4 exhibited a synergistic anticancer effect on gastric cancer MGC803 cells [123]. They distinctly induced intracellular ROS generation, reduced the mitochondrial transmembrane potential ($\Delta\Psi_m$), enhanced the expression of caspase-3, and decreased the expression of BCL-2. As TQ has strong anticancer activity, whether the combination of berberine and d-limonene exerted synergistic anticancer effects merits study [123].

4.2. Metabolism

Rabbit carcinogen metabolizing enzymes CYP1A2, 3A4, but not CYP2E1, were significantly diminished by dietary doses of thymoquinone [124]. Thymoquinone displays a significant inhibition of induced phase I CYP1A1 enzyme, and increases the content of glutathione and activity of phase II enzyme glutathione-S-transferase, in HCC HepG2 cells, providing support for the beneficial use of thymoquinone as a therapeutic and chemopreventive agent against liver cancer, as reviewed in [125]. The phase II enzyme glutathione peroxidase was also significantly induced by the high thymoquinone dose in rabbit, while the total glutathione levels were unaffected [124]. Glutathione reductase was significantly induced, which may explain the benign effect of *N. sativa* seeds in inhibiting the generation of bioactive metabolites known to promote carcinogenesis and oxidative cell damage [124, 125].

In human P-glycoprotein (MDR1, ABCB1) gene-transfected KB/MDR1 cells, TQ had no effect on the accumulation of daunorubicin or rhodamine-123, two fluorescent substrates of MDR1 [126]. Acerinol, a cyclolanstane triterpenoid from *Cimicifuga acerina*, could increase the chemosensitivity of MDR1-overexpressing HepG2/ADM and MCF-7/ADR cells to chemotherapeutic drugs doxorubicin, vincristine, and paclitaxel [127]. It could also increase the retention of MDR1 substrates doxorubicin and rhodamine-123 in the above cells [127]. Acerinol significantly stimulated the activity of MDR1 ATPase without affecting the expression of MDR1 on mRNA or protein level [127]. Acerinol reversed the resistance of MCF-7/ADR cells to vincristine [127]. Acerinol may be a competitive inhibitor of MDR1, and docking analysis data indicated that acerinol would bind to the sites on MDR1 that partially overlapped with that of verapamil [127].

The ethanolic *Cimicifuga racemosa* extract BNO-1055, rich in saponin, dose-dependently attenuated a cellular uptake and incorporation of thymidine and bromodeoxyuridine (BrdU) and significantly inhibited cell growth after long-time exposure [128]. These inhibitory effects of BNO-1055 could be mimicked using pharmacological inhibitors and isoform-specific siRNAs targeting the equilibrative nucleo-

side transporters, ENT1 and 2, as indicated in [128]. BNO-1055 also attenuated the uptake of clinically relevant nucleoside analogs, e.g. the anticancer drugs gemcitabine and fludarabine [128]. By inhibiting the salvage nucleoside uptake pathway, BNO-1055 potentiated the cytotoxicity of the *de novo* nucleotide synthesis inhibitor 5-fluorouracil without significantly altering its uptake [128]. In human breast cancer MCF-7 cells, both *C. racemosa* extract and purified cycloartane saponins upregulated the expression of CYP1A1 and CYP1B1, two carcinogen-metabolizing enzymes, as described elsewhere [9].

Aconite root could improve the energy metabolism in rats, by influencing the metabolic process of sugar, lipid and amino acid, which may be the main molecular mechanism of warming yang and dispelling cold for the treatment of the cold syndrome, common in cancer patients, according to TCM theory, as reviewed in [129]. *A. napellus* extract administered around the clock induced hyperthermia overall and in a time-dependent manner, with greatest effects during the resting span [130]. Thus, time of day may significantly affect the outcome of *A. napellus* and other homeopathic treatments and should be considered in determining optimal dosing and treatment time in order to increase the desired outcome and decrease undesired effects [130]. Fu Zi (lateral roots of *A. carmichaelii*) can increase and maintain the dogs' body heat for at least 6 h [131]. The body weight gain in aconitine-administered mice was less than that of the control group until day 22, as described in [132]. Transient rectal hypothermia occurred within 30 min after the last administration of aconitine [132]. Then the rectal temperature gradually increased to normal [132]. The drug metabolism of aconitine increased and the toxicity of aconitine decreased due to long-term administrations of aconitine [132].

The crude extract of secondary roots of *Aconitum carmichaelii* Debeaux (Fu Zi), together with its processed products, including Yanfuzi, Heishunpian and Paofupian, are commonly applied in clinic for various biomedical purposes [133]. Fingerprints of Fu Zi, Yan Fu Zi (salted prepared aconite root), Heishunpian (processed Fu Zi) and Paofupian were obtained by ultra-high performance liquid chromatography (UPLC) and their effects on mitochondrial metabolism were studied by microcalorimetry [133]. Due to inherent differences in chemical compositions, the energy metabolism of mitochondria was variably influenced by selected Fu Zi and its processed products [133]. The bioactivity sequence of the tested products was Fu Zi > Heishunpian > Paofupian > Yan Fu Zi, as indicated in [133]. The phytometabolites mesaconitine (Fig. 3, structure 20), benzoilaconitine, and benzoylhypaconitine might be the principal active components that modulate energy metabolism [133].

Reprogramming energy metabolism is an emerging cancer hallmark [134]. The cancer cells rely on aerobic glycolysis, instead of mitochondrial oxidative phosphorylation, to yield ATP [134]. The enhanced glycolysis allows the diversion of glycolytic intermediates into various biosynthetic pathways, including those generating nucleosides and amino acids, which facilitates the biosynthesis of the macromolecules and organelles required for assembling new cells [134]. Can *Aconitum* reverse the deregulated cellular energetics of cancer cells? The TCM formula Sini decoction (SND), con-

sisting of *Fu Zi*, *Glycyrrhizae Preparata Radix* and *Zingiberis Rhizoma*, significantly inhibit LDH and glycolysis in the myocardial ischemia reperfusion injury model of rat H9c2 cardiomyocytes [135]. SND also modulates lipid metabolism, tricarboxylic acid cycle and nitrogen metabolism [135]. It is intriguing to study whether *Aconitum* and diterpenoid alkaloids thereof inhibit the aerobic glycolysis of cancer cells [135]. To date there is also no direct evidence that *Aconitum* enhances mitochondrial oxidative phosphorylation in human cancer cells [135].

5. ANTI-ANGIOGENIC AND ANTI-METASTATIC EFFECTS

5.1. Saponins

SB365 exhibited potent anti-angiogenic activity and decreased the expression of hypoxia-inducible factor-1 α (HIF-1 α) and vascular endothelial growth factor (VEGF), a key molecule for angiogenesis [21]. SB365 suppressed the tube formation and migration of human umbilical vein endothelial cells (HUVECs), as well as *in vivo* neovascularization in a mouse Matrigel plug assay [21]. SB365 treatment decreased the expressions of VEGF and CD34 in the tumor tissue of HCC xenograft models [21]. Angiogenesis of human colon cancer and pancreatic cancer cells was also suppressed by SB365 [22, 23]. SB365 inhibited tube formation in hepatocyte growth factor (HGF)-induced HUVECs and suppressed micro vessel sprouting from the rat aortic ring, *ex vivo*, and blood vessel formation in the Matrigel plug assay in mice [27].

Raddeanin A significantly inhibited HUVEC proliferation, motility, migration, and tube formation [30]. Raddeanin A dramatically reduced angiogenesis in chick embryo chorioallantoic membrane, restrained the trunk angiogenesis in zebrafish, and suppressed angiogenesis and growth of human colorectal cancer HCT-15 cell xenografted into mice [30]. Raddeanin A suppressed VEGF-induced phosphorylation of VEGF receptor-2 and its downstream protein kinases including PLC γ 1, JAK2, FAK, SRC, and AKT, as indicated in [30]. In molecular docking simulation, Raddeanin A formed hydrogen bonds and hydrophobic interactions within the ATP-binding pocket of VEGF receptor-2 protein kinase domain [30]. Raddeanin A significantly inhibited the invasion, migration and adhesion of the BGC-823 human gastric cancer cells [31]. Raddeanin A could upregulate the expression of REversion inducing Cysteine rich protein with Kazal motifs (RECK) and E-cadherin proteins, and down-regulate the expression of matrix metalloproteinase-2 (MMP-2), MMP-9, MMP-14 and RhoC proteins, as described in [31].

5.2. Terpenoid

Thymoquinone inhibits cancer angiogenesis, cell invasion, and metastasis [11, 136]. TQ treatment decreased the transcriptional activity of the TWIST1 promoter and the mRNA expression of TWIST1, a transcription factor promoting epithelial-to-mesenchymal transition (EMT), as indicated in [137]. Thymoquinone also decreased the expression of TWIST1-upregulated genes, such as N-cadherin and increased the expression of TWIST1-repressed genes such as E-cadherin, thus reducing cell migration and invasion [137]. Thymoquinone inhibited the growth and metastasis of cancer

cell-derived xenografted tumors in mice and partially attenuated the migration and invasion of TWIST1-overexpressed cell lines [137]. Furthermore, thymoquinone enhanced the promoter DNA methylation of the TWIST1 gene in human breast cancer BT-459 cells [137].

Cimyunnin A (12 of Fig. 1), a triterpene isolated from the fruit of *Cimicifuga yunnanensis* and characterized by an unusual fused cyclopentenone ring G, exhibited comparable anti-angiogenic activities to those of sunitinib, a clinically-used first-line angiogenesis inhibitor, in the *in vitro* and *ex vivo* studies [138]. The phosphorylation of VEGF receptor-2 protein was suppressed by cimyunnin A in a dose-dependent manner [138]. Dramatic downregulation of p-AKT (Ser473) and p-ERK (Thr202/Tyr204), the known downstream targets of VEGF receptor 2, were observed at 5.0 and 10.0 μ M of cimyunnin A, while total VEGF receptor 2, ERK, and AKT remain unchanged [138].

5.3. Alkaloid

Isoquinoline alkaloids such as berberine, palmatine, jatrorrhizine, and columbamine are abundant in *Coptis*, *Thalictrum*, and *Hydrastis*, and exert their anti-cancer activity via multiple molecular mechanisms [139]. Berberine inhibited colorectal cancer invasion and metastasis via down-regulation of COX-2/PGE2-JAK2/STAT3 signaling pathway [140]. The MMP-1, -2, and -9 expressions were also decreased by berberine, while MMP-7 was not affected [121, 140]. Berberine inhibits the metastatic ability of prostate cancer cells by suppressing EMT-associated genes, e.g., BMP7, NODAL, and SNAIL, which, if highly expressed, are associated with shorter survival of prostate cancer patients. Berberine inhibited the expression of Id-1 (inhibitor of differentiation/DNA binding), a key regulator of HCC development and metastasis [141, 142]. Berberine could inhibit the *ID-1* gene promoter activity [142]. The ID-1 protein overexpression in HCC models partially rescued anti-proliferative and anti-invasive activities of berberine. Purified palmatine, or in the extract, exhibits invasion inhibitory properties, as reviewed elsewhere [139]. Synergistic inhibition of the ribosomal protein S6 (RPS6)/NF- κ B/FLIP axis with palmatine may have therapeutic potential for cancer with their constitutive activation, as reviewed in [139].

Anoikis, or detachment-induced apoptosis, may prevent cancer progression and metastasis by blocking signals necessary for survival of localized cancer cells, as reviewed in [143]. Resistance to anoikis is regarded as a precondition for metastasis [143]. Berberine promoted the growth inhibition of anoikis-resistant cells to a greater extent than doxorubicin treatment [143]. Berberine induced cell cycle arrest at G0/G1 in the anoikis-resistant MCF-7 and MDA-MB-231 breast cancer cells [143].

Jatrorrhizine induced C8161 metastatic melanoma cell cycle arrest at the G0/G1 transition, which was accompanied by overexpression of the cell cycle inhibitor proteins, CDKN1A (p21) and CDKN2B (p27), at higher doses [144]. Jatrorrhizine failed to induce a significant cellular apoptosis at doses up to 320 μ mol/l, as indicated [144]. Moreover, jatrorrhizine reduced C8161 cell-mediated neovascularization *in vitro* and *in vivo* and downregulated the expression of cadherin, a key protein in tumor vasculogenic mimicry and angiogenesis [144].

Columbamine induces metastatic osteosarcoma U2OS cell cycle arrest at the G2/M transition, which is associated with attenuating CDK6 gene expression and diminishing STAT3 phosphorylation, as described in [145]. Columbamine did not significantly promote cell apoptosis at any dosages tested [145]. Columbamine inhibited U2OS cell-mediated neovascularization, which was accompanied by the down-regulation of MMP-2 expression and reduction of cell migration, adhesion, and invasion [145].

5.4. Plant Extract

Pulsatilla koreana extract decreased the expression of HIF-1 α and VEGF in HCC cells, and inhibited tube formation and migration of HUVEC, as described elsewhere [86]. *Pulsatilla koreana* extract potently suppressed *in vivo* neovascularization in a mouse Matrigel plug assay. In a mouse xenograft model, the expressions of Ki-67, VEGF, and CD31 in the tumor tissue were decreased by *Pulsatilla koreana* extract [86]. Angiogenesis of anaplastic thyroid cancer was also suppressed by *Pulsatilla koreana* extract [86]. The water extract of *Pulsatilla koreana*, *Panax ginseng* (Ren Shen in TCM) and *Glycyrrhiza uralensis* (Gan Cao in TCM) (WEPPG) significantly inhibited fibroblast growth factor-induced HUVEC proliferation, adhesion, migration, and capillary tube formation [146]. The protein levels of cyclin A (CCNA), TP63 and CDKN1C (also known as p27KIP2) were upregulated, while nibrin and focal adhesion kinase (FAK) were downregulated [146]. The blood vessel formation in a CAM treated with WEPPG was markedly reduced. WEPPG might exert its anticancer effects via the inhibition of angiogenesis [146].

Aconite from *Aconiti Kusnezoffii Radix* in TCM could induce cell differentiation, inhibit cell proliferation and migration, increase succinate dehydrogenase (SDH) activity and promote gap-junction intercellular communication in Lewis lung cancer cells [147]. Extract from *Coptidis Rhizoma* increases cell adhesion and decreases SDH activity and GJIC without cell differentiation while it also suppressed cell proliferation [147]. *Aconiti Kusnezoffii Radix* water decoction could maintain body temperature, blood oxygen saturation, red cell ATP-ase and blood rheology, and decrease intratumor hypoxia and capillary permeability in tumor-bearing mice, which retarded cancer growth and metastasis [147]. Extract from *Aconitum vaginatum* can inhibit the proliferation, invasion and metastasis of A549 cells, and MMP-2 and -9 activities were decreased [148]. *Coptidis Rhizoma* water decoction decreased body temperature, blood oxygen saturation, red cell ATPase, blood rheology and gap-junction intercellular communication, and promoted intratumor hypoxia and capillary permeability, which led to more cancer metastasis although it also inhibited cancer growth [147, 149]. The hot Chinese medicine (*e.g.*, aconite) could induce cancer cell differentiation and prevent tumor invasion, which might be better for lung cancer treatment than cold Chinese medicine (*e.g.*, *Coptidis Rhizoma*), as indicated in [147, 149].

Coptidis rhizome aqueous extract (CRAE), containing magnoflorine (17 of Fig. 1) 2.2%, jatrorrhizine 1.68%, palmatine 4.4%, and berberine 13.8%, exhibited significant inhibition on VEGF secretion from MHCC97L and HepG2

cells at nontoxic doses [149]. CRAE intervention increased the phosphorylation of eukaryotic elongation factor 2 (eEF2) in HCC cells, which blocked eEF2 activity for proceeding nascent protein synthesis [149]. Reduction of tumor size and neovascularization were observed in mice xenograft model [149].

6. IMMUNOMODULATORY ACTIVITY

The immune destruction of cancer cells is an essential anticancer mechanism, as defined in [134]. *Ranunculus ternatus* polysaccharides enhance the proliferation of mouse thymocytes, spleen lymphocytes, and peritoneal macrophages [150]. *R. ternatus* polysaccharides enhance NK cell activity and induce apoptosis in breast cancer MCF-7 cells [151, 152]. However, the anti-proliferative effects of *R. ternatus* polysaccharides might be less pronounced than saponins in human gastric cancer BGC823 cells [151, 152]. *Pulsatilla chinensis* polysaccharides (PCPS) treatment to glioma-bearing mice could elevate the thymus and spleen indices [106]. A neutral polysaccharide fraction (ARP) from the rhizome of *Anemone raddeana* extraordinarily promotes splenocyte proliferation, NK cell and CTL activity, as well as serum IL-2 and TNF- α production in HCC-bearing mice [153]. ARP had no toxicity to body weight, liver, and kidney [153]. Moreover it could reverse the hematological parameters induced by 5-fluorouracil to near normal [153]. A polysaccharide (ACP-a1) of 3.2 $\times 10^5$ Da, was isolated from the roots of *Aconitum coreanum*, and is mainly composed of β -D-mannose and β -D-glucose in a molar ratio of 1.2:3.5, as indicated in [154]. ACP-a1 significantly inhibited the growth of hepatoma H22 transplanted in mice and prolonged the survival time of H22 tumor-bearing mice [154]. The body weight, peripheral white blood cells, thymus index and spleen index of H22 tumor-bearing mice were improved after ACP-a1 treatment [154]. ACP-a1 could promote the secretion of serum cytokines, such as IL-2, TNF- α and IFN- γ , as described in [154]. *Nigella sativa* is highlighted as a natural immune booster [155]. Modes of its actions include boosting and functioning of immune system, activation and suppression of immune specialized cells, interfering in several pathways that eventually lead to improved immune responses and defense system [155]. For instance, thymoquinone induces the phosphorylation of proteins involved in NK cell signaling, CD28 signaling of T_H cells, and B cell receptor signaling [156, 157].

7. ANTI-INFLAMMATORY ACTIVITY

Inflammation has been identified as a significant factor in the development of solid tumors [103, 134]. For instance, both hereditary and sporadic forms of chronic pancreatitis are associated with an increased risk of developing pancreatic ductal adenocarcinoma (PDA), as indicated in [103]. Thymoquinone, the major constituent of *Nigella sativa* oil extract, induced cell cycle arrest and apoptosis in PDA cells via an increased CDKN1A (p21WAF1) expression, decreased HDAC activity, and induced histone hyperacetylation [103]. Thymoquinone was also shown to reduce expression of monocyte chemoattractant protein (MCP)-1, tumor necrosis factor (TNF)- α , interleukin-1 β and COX-2 in PDA cells in a dose- and time- dependent fashion [103]. Thymoquinone also inhibited the constitutive and TNF- α mediated

activation of NF- κ B and reduced its transport from the cytosol to the nucleus in PDA cells [103]. Similar to berberine, the anti-inflammatory activity of thymoquinone might contribute to its overall anti-cancer effects [155, 158].

Ranunculaceae tribes and genera, such as *Ranunculus*, *Anemoneae*, *Cimicifuga*, *Helleborus*, *Nigella*, *Delphinieae*, *Semiaquilegia*, *Coptis*, and *Hydrastis*, are rich in both anti-inflammatory and anticancer phytometabolites [1, 2]. Anemonin (24 of Fig. 1) and ranunculin (25 of Fig. 1), the potent anti-inflammatory and anti-cancer phytometabolites, are abundant in tribes Ranunculaceae and Anemoneae [2, 159]. The chronic inflammation is associated with tumor formation, and tumors could be portrayed as wounds that never heal [160]. *Ranunculus pedatus* and *R. constantinopolitanus* were found to have wound healing and anti-inflammatory properties [161]. The fatty-acid fractions of *R. constantinopolitanus* exerted the anti-inflammatory effects via down-regulation of interleukin-6 and COX-2, as indicated in [162]. The triterpene saponins, phenolic acid, lignans, and flavonoids of *Clematis* display various anti-inflammatory activities [1, 2, 7]. *C. mandshurica* extract inhibited interleukin-1 β -induced MMP gene expression, implicating its use in combating against cancer invasion [163]. The inflammatory cells can release chemicals, notably ROS, which are actively mutagenic for nearby cancer cells, accelerating their genetic evolution toward states of intensified malignancy [164]. The *Ranunculaceae* anti-inflammatory compound remarkably inhibited ROS generation and downregulated the ROS-dependent NF- κ B signaling pathway [165].

Cycloartane-type triterpene saponins (*Cimicifuga* and *Actaea*), diterpenoid alkaloids (*Aconitum* and *Delphinium*), flavonoid glycosides (*Ranunculus* and *Trollius*), and isoquinoline alkaloids (subfamily *Thalictrioideae*, *Coptis*, and *Hydrastis*) also display various anti-inflammatory activities [2, 166]. The cytokines (e.g. IL-6 and IFN- γ) and chemokines (e.g. MIP-1 β) were decreased in serum of MCS-18 (from *Helleborus*)-treated mice [167]. *Dichocarpum* (firstly identified as an independent genus by Xiao and Wang in 1964), *Adonis*, *Beesia*, *Caltha*, and *Halerpestes* are less studied in modern pharmacology, although in folk medicine and TCM they are frequently used in the inflammatory diseases [2, 168]. Taken together, *Ranunculaceae* plants contribute notable chemodiversity, from which promising anticancer phytometabolites and novel anticancer mechanisms could be found, as reviewed elsewhere [2].

8. STRUCTURE-ACTIVITY RELATIONSHIP

Saponins 1-14 of *P. chinensis* showed considerable cytotoxic activity, whereas saponins 15-36 had no significant activity, suggesting that a free carboxylic group at C-28 of aglycon is essential for their cytotoxic activity [169]. The oleanane-type saponins showed better cytotoxic activity than lupane-type saponins, and the length and linkage of glycolic chain attached to C-3 of aglycon displayed an important effect to the cytotoxic potency [169]. Oleanolic acid 3-O- α -L-rhamnopyranosyl-(1 \rightarrow 2)-[β -D-glucopyranosyl-(1 \rightarrow 4)]- α -L-arabinopyranoside (saponin 5) exhibited the most significant cytotoxic activity [169].

Saponins 5-17 of *P. koreana*, with a free acidic functional group at C-28 of aglycon, exhibited moderate to con-

siderable cytotoxic activity, while saponins 1-4, esterified with a trisaccharide at C-28 of aglycon, did not exhibit cytotoxic activity ($ED_{50} > 300 \mu\text{M}$). Oleanolic acid 3-O- α -L-rhamnopyranosyl-(1 \rightarrow 2)-[β -D-glucopyranosyl-(1 \rightarrow 4)]- α -L-arabinopyranoside (saponin 10) exhibited the most potent cytotoxic activity [170]. During *in vivo* studies, hederagenin 3-O- α -L-rhamnopyranosyl-(1 \rightarrow 2)-[β -D-glucopyranosyl-(1 \rightarrow 4)]- α -L-arabinopyranoside (saponin 6) exhibited more potent anticancer activity than paclitaxel and doxorubicin [170]. Hederagenin 3-O- β -D-glucopyranosyl-(1 \rightarrow 4)-O- β -D-glucopyranosyl-(1 \rightarrow 3)-O- α -L-rhamnopyranosyl-(1 \rightarrow 2)- α -L-arabinopyranoside (saponin 17) exhibited potent anticancer activity [170]. These two saponins were similarly comprised of a hederagenin aglycon and a sugar sequence O- α -L-rhamnopyranosyl-(1 \rightarrow 2)- α -L-arabinopyranoside at C-3 of the hederagenin, suggesting that the two elements are essential for the anticancer activity [170].

The natural and derivatized C_{19} -diterpenoid alkaloids of *Aconitum* tested by Hazawa *et al.* [171] and Wada *et al.* [172] showed only a slight or no effect against cancer cells. Most anticancer phytometabolites were hetisine-type C_{20} -diterpenoid alkaloids, specifically kobusine and pseudokobusine analogs with two different substitution patterns, C-11 and C-11, 15, as indicated in [173]. Particularly, several C_{20} -diterpenoid alkaloids were more potent against multidrug-resistant KB subline KB-VIN cells [173]. Pseudokobusine 11-3'-trifluoro-methylbenzoate (19 of Fig. 1) is a possible promising new lead meriting additional evaluation [173].

9. GENOMICS, TRANSCRIPTOMICS, PROTEOMIC, AND METABOLOMICS

It is becoming increasingly apparent that a precise and truly useful understanding of the behavior of individual phytometabolite and *Ranunculaceae* extracts would only come if we are able to integrate genomic, proteomic, and other omics datasets, and then distill these data. The use of omics platforms in *Ranunculaceae* anti-cancer research is still in its infancy. However, initial omics information is schematically summarized in (Fig. 5). For instance, a PCR expression array was used to quantify thymoquinone-mediated transcriptional regulation of 84 apoptosis-related genes [174]. At low dose (12.5 μM), thymoquinone induced expression of four pro-apoptotic genes: BIK (~22.7-fold), FASL (~2.9-fold), BCL2L10 (~2.1-fold), and CASP1 (~2-fold), as described elsewhere [174]. Thymoquinone reduced the expression of an anti-apoptotic gene implicated in NF- κ B signaling and cancer: RELA (~8-fold), as described in [174]. At high dose (100 μM), thymoquinone mediated the expression of 21 genes directly implicated in apoptosis (6 genes), TNF signaling (10 genes), and NF- κ B signaling (3 genes), as indicated in [174]. They include: BIK, BID, TNFRSF10A, TNFRSF10B, TNF, TRAF3, RELA, and RELB [174]. Thus, these data support the notion that thymoquinone intervenes with TNF and NF- κ B signaling during thymoquinone-mediated induction of apoptosis in cancer cells [174].

DNA microarray data for 12,600 genes were used to examine the anti-proliferative activity of *Coptidis rhizoma* and eight constituent molecules against eight human pancreatic cancer cell lines [175]. Twenty-seven genes showed a strong

correlation with the 50% inhibitory dose (ID₅₀) of *Coptidis rhizoma* after 72-h exposure [175]. The test molecules were classified into two clusters, one consisting of *Coptidis rhizoma* and berberine, and the other consisting of the remaining seven molecules [175]. Berberine can account for the majority of the anti-proliferative activity of *Coptidis rhizoma* and DNA microarray analyses can be used to improve our understanding of the actions of an intact herbal compound [175].

Identifying human or mammalian metabolites is an integral part of the molecular mechanism investigations of anti-cancer phytometabolites. Eighteen metabolites of *Pulsatilla* saponin D were identified in rat plasma, urine and feces samples by ESI-Q-TOF-mass spectrometry (MS/MS), and eight of them (M11-M18) were novel ones [26]. Deglycosylation, dehydrogenation, hydroxylation, and sulfation were the major metabolic transformations of *Pulsatilla* saponin D *in vivo* [26]. The metabolic information gained from metabolomic studies is relevant to the pharmacological activity of *Pulsatilla* saponin D, as reviewed in [26].

Bioinformatics and chemoinformatics are the indispensable part of omics platforms that contribute prolific information of molecular mechanisms of anti-cancer phytometabolites. Three different *in silico* reverse screening approaches proved useful for identifying the putative molecular targets of the anti-cancer phytometabolites in *Nigella sativa* (thymoquinone, α -hederin, dithymoquinone, and thymohydroquinone), as indicated in [176]. Novel kinase targets influenced by thymoquinone were revealed by *in silico* analysis of peptide array data obtained from thymoquinone-treated HCT116 colon cancer cells [57]. Thymoquinone induces the phosphorylation of a multitude of proteins that are involved in one or more cancer related biological functions: apoptosis, proliferation and inflammation [57]. Of the 104 proteins identified, 50 were upregulated ≥ 2 fold by thymoquinone and included molecules in the AKT-MEK-ERK1/2 pathway [57]. Oncogenic PAK1 emerged as an interesting thymoquinone target [57]. Thymoquinone induced an increase of pERK1/2 and triggered the early formation of an ERK1/2-PAK1 complex [57]. Modeling confirmed that thymoquinone binds in the vicinity of Thr212 accompanied by conformational changes in ERK2-PAK1 binding [57]. Structural modeling analysis suggests that thymoquinone interferes also with the kinase domain and disturbs its interaction with p-PAK (Thr423), finally inhibiting MEK-ERK1/2 signaling and disrupting its prosurvival function [57].

Using cDNA microarray technology, thymoquinone was found to alter expression of 577 genes (that showed >2 -fold change in expression) in human breast cancer MCF7 cells, as described elsewhere [177]. Of these genes, 45.2% showed an upregulation and 54.7% showed a downregulation in expression [177]. The cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1) and UDP glucuronosyltransferase 1 family, polypeptide A8 (UGT1A8) genes were downregulated significantly by 43- and 11-fold, respectively [177]. The solute carrier family 7, member-11 (SLC7A11) gene was downregulated by 15-fold [177]. The interferon-induced protein with tetratricopeptide repeats (IFIT)-1, IFIT2, IFIT3, interferon, α -inducible protein (IFI)-6 (also known as G1P3), interferon regulatory factor 9 (IRF9, ISGF3), 2'-5'

oligoadenylate synthetase 1 (OAS1) and signal transducer and activator of transcription-1 (STAT1) genes showed changes in expression following treatment with thymoquinone [177]. The caspase-10 (CASP10) gene was activated and the protein tyrosine phosphatase, receptor type R (PTPRR) and myocyte enhancer factor 2C (MEF2C) genes were upregulated [177]. These findings indicate that thymoquinone targets specific genes in the estrogen metabolic and interferon pathways [177].

10. CONCLUSION AND FUTURE PERSPECTIVE

This review focuses on the various *Ranunculaceae* chemical phytometabolites that have shown promise as anti-cancer agents and outlines their potential mechanism of action. Deeper insights into molecular mechanisms and functions of *Ranunculaceae* anti-cancer phytometabolites are the premise of designing analog and nanoformulation with improved absorption, distribution, metabolism, and excretion/toxicity properties and therapeutic efficacy. Studies of the representative *Ranunculaceae* phytometabolites, e.g., thymoquinone and berberine, are enlightening, since other phytometabolites of the same plant family could share many anti-cancer mechanisms and pathways. To date, little information has been reported on the anticancer effects and the underlying mechanisms of the diterpenoid alkaloids of *Aconitum* and *Delphinium* plants. Many *Ranunculaceae* phytometabolites have shown very promising anti-cancer properties *in vitro*, but have yet to be evaluated in humans. The potential role for *Ranunculaceae* phytometabolites in epigenetic regulation of gene expression remains largely unknown so far. Gaps are present in the extensive use of high-throughput transcriptome sequencing and proteome profiling for characterizing the interaction between *Ranunculaceae* phytometabolites and cancer cells, as well as the relevant regulatory network. Further studies are warranted to determine the molecular mechanisms and efficacy of *Ranunculaceae* phytometabolites in treating human cancers.

Many *Ranunculaceae* plants contain not only anti-cancer phytometabolites but also phytometabolites with free radical scavenging, immunomodulatory, and anti-inflammatory activities that are helpful against cancer insurgence. However, interaction between western drugs and herbs/botanicals should be well investigated before safe combined use, and such information must be disseminated to the allied stakeholders. As anti-cancer candidates with low toxicity, *Ranunculaceae* phytometabolites, such as thymoquinone and berberine, and their altered structure, as well as source plants such as *N. sativa*, *C. chinensis* and their closely related species, will attract researchers to pursue the potential anticancer effects and the mechanisms by using innovative technologies of genomics, proteomics and other advanced approaches. Current and future studies would consistently suggest the feasibility of mining anticancer pharmacological diversity from *Ranunculaceae* chemodiversity.

LIST OF ABBREVIATIONS

ALT	= Alanine aminotransferase
AMPK	= AMP-activated protein kinase
APAF-1	= Apoptotic peptidase activating Factor 1

AST	= Aspartate aminotransferase	NF-κB	= Nuclear factor kappa B
ATM	= Ataxia telangiectasia mutated	OAS1	= 2'-5'-oligoadenylate synthetase 1
BIRC5	= Baculoviral inhibitor of apoptosis protein repeat-containing protein	p70S6K	= p70S6 kinase
CAT	= Catalase	PDA	= Pancreatic ductal adenocarcinoma
CDC2	= Cell division cycle protein 2 homolog	PTEN	= Phosphatase and tensin homolog
CHK	= Checkpoint kinase	PI3K	= Phosphoinositide 3-kinase
CDK1	= Cyclin-dependent kinase 1	PBD	= Polo-box domain
CRAE	= Coptidis rhizome aqueous extract	PLK	= Polo-like kinase
COX-2	= Cyclooxygenase-2	PARP	= Poly-ADP ribose polymerase
CYP1A1	= Cytochrome P450, family 1, subfamily A, polypeptide 1	PTPRR	= Protein tyrosine phosphatase, receptor type R
DIME	= Dihydroxy-isosteviol methyl ester	PCPS	= Pulsatilla chinensis polysaccharides
DNA-PK	= DNA-dependent protein kinase	RPS6	= Ribosomal protein S6
DNMT	= DNA methyltransferase	QR	= Quinone reductase
DRβ-H	= D-Rhamnose β-hederin	ROS	= Reactive oxygen species
ER	= Endoplasmic reticulum	RTK	= Receptor tyrosine kinase
EGF	= Epidermal growth factor	RECK	= REversion inducing Cysteine rich protein with Kazal motifs
EMT	= Epithelial-to-mesenchymal transition	STAT1	= Signal transducer and activator of transcription-1
EMMPRIN	= Extracellular matrix metalloproteinase inducer	SLC7A11	= Solute carrier family 7, member-11
eEF2	= Eukaryotic elongation factor 2	SAHA	= Suberoylanilide hydroxamine
FAK	= Focal adhesion kinase	SDH	= Succinate dehydrogenase
FOXO	= Forkhead box	SOD	= Superoxide dismutase
GBM	= Glioblastoma multiforme	TQ	= Thymoquinone
HCC	= Hepatocellular carcinoma	TCM	= Traditional Chinese medicine
HGF	= Hepatocyte growth factor	TOP	= Topoisomerase
HDAC	= Histone deacetylase	TNF	= Tumor necrosis factor
HUVEC	= Human umbilical vein endothelial cells	TP	= Tumor protein
HIF-1α	= Hypoxia-inducible factor-1α	XIAP	= X-linked inhibitor of apoptosis protein
IAP	= Inhibitors of apoptosis	VEGF	= Vascular endothelial growth factor
IFIT	= Interferon-induced protein with tetratricopeptide repeats	UGT1A8	= UDP glucuronosyltransferase 1 family, polypeptide A8
IFI	= Interferon, α-inducible protein	CDK	= Cyclin dependent kinase
IRF	= Interferon regulatory factor	DSB	= Double-strand break
IP3	= Inositol 1,4,5-trisphosphate receptor	eEF	= Eukaryotic elongation factor
IGF	= Insulin-like growth factor	EGFR	= Epidermal growth factor receptor
JNK	= c-Jun NH ₂ -terminal kinase	EMT	= Epithelial to mesenchymal transition
mTOR	= Mammalian target of rapamycin	ER	= Estrogen receptor
miR	= microrna	ERK	= Extracellular signal-regulated kinase
MAPK	= mitogen-activated protein kinase	HCC	= Hepatocellular carcinoma
MCP	= monocyte chemoattractant protein	HGF	= Hepatocyte growth factor
MPM2	= M-phase phosphoprotein-2	HIF-1α	= Hypoxia-inducible factor 1α
MEF2C	= Myocyte enhancer factor 2C	HUVEC	= Human umbilical vein endothelial cell
		JNK	= c-Jun NH ₂ -terminal kinase

MAPK	=	Mitogen-activated protein kinase
mTOR	=	Mammalian target of rapamycin
PARP	=	Poly-ADP ribose polymerase
SND	=	Sini decoction
TCM	=	Traditional Chinese medicine
UPLC	=	Ultra-high performance liquid chromatography
VEGF	=	Vascular endothelial growth factor

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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