Chapter 02

Literature Survey

2 Chapter 02: Importance of natural product molecules and targeted drug delivery

2.1 Natural products as a foundation for drug discovery

Nature serves as an inherent biochemical facility, generating both primary and secondary metabolites in response to the complex biological needs it encounters. Primary metabolites play a crucial role in cell reproduction and metabolism, while secondary metabolites, such as terpenes, glycosides, alkaloids etc. exhibit specific functionalities like anticancer, antibacterial and other properties within an organism. Phytochemical components like alkaloids, terpenoids, flavonoids, phenolics, essential oils, tannins, and saponins, renowned for their bioactive characteristics, emerge as vital byproducts of medicinal plants.

Modern medicine, as well as traditional medicine, primarily originate from natural substances. The discovery of novel biologically active molecules from sources such as plants, bacteria, and marine organisms has spurred the development of innovative natural drugs. Compounds derived from nature exhibit enhanced drug properties, with examples such as 65% of substances from the natural product dictionary complying with Lipinski's rule. Additionally, natural products containing metabolites demonstrate greater potency in comparison to artificially synthesized compounds (55).

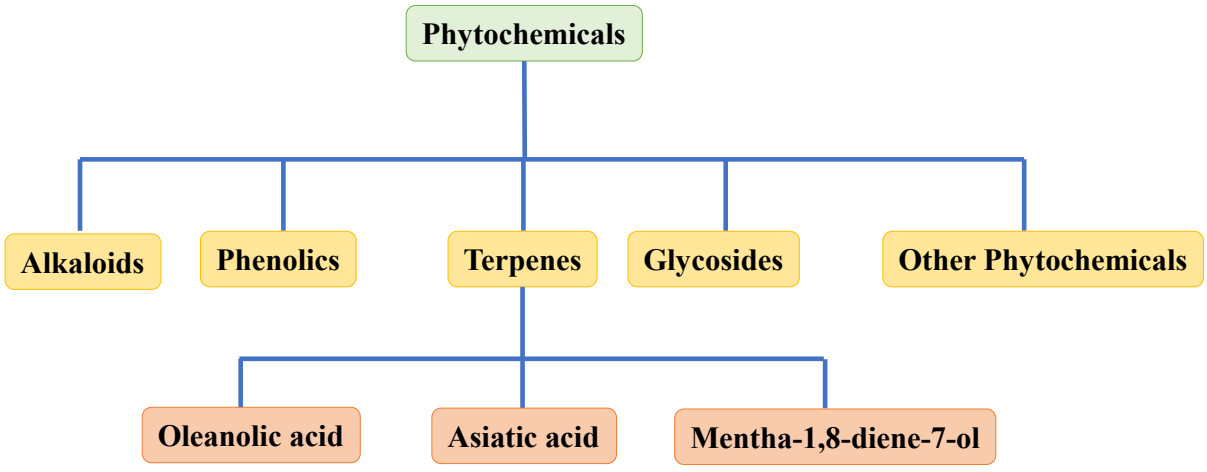


Figure 5 Bioactive natural products

The need for novel medications has led to the exploration of new natural products, given their significant medicinal value. Between 2019 and 2020, 30% of the most successful drugs were directly derived from natural products, including their derivatives and medications related to their active core moiety (56, 57).

2.2 Natural product drug discovery

From 1981 to 2019, the USFDA approved a total of 1881 new drugs, referred to as new chemical entities (NCEs). Among these, 930 new products associated with natural medicines were introduced to the market during the same period, with 71 drugs directly originating from natural products (58). Until 1946, the USFDA granted approval to only four directly sourced natural products and one derived from a natural product. The count of unaltered natural products or those minimally modified increased from 1981 to 1987. The peak occurred in 1987, with the approval of the highest number (78) of new chemical entities as drugs, out of which 32 were natural products and natural product-related drugs. Conversely, the lowest number of new drugs (24) received approval in 2004, with 37% (9) falling into the category of natural products and their derivatives. The approval of new natural product derivatives witnessed an upward trend from 1976 to 2019, with 37 new active substances derived from natural products between 2006 and 2015 (59).

2.2.1 Analysis of natural product sources

A sum of 279 compounds has been recognized as drugs derived from natural products and their derivatives as of 2015. Out of these, 102 drugs (37%) were directly sourced from plant products, 93 drugs (33%) were isolated from bacterial sources, 72 drugs (26%) originated from marine organisms, and 5 molecules (2%) had different origins. It's noteworthy that, with the exception of dicoumarol, all drugs were direct natural products until 1946. Between 1946 and 1965, the USFDA granted approval to an average of nearly 1.7 plant-based natural product drugs per year. Subsequently, the approval rate declined to less than one per year from 1986 to

2015 (60). Between 1946 and 1955, fifteen compounds originated directly from natural products, while 11 compounds were derived from natural products and entered the market. From 1956 to 1965, a total of 32 compounds received approval from the USFDA as new drugs, with 20 of them being natural products and the remaining 12 being derivatives of natural products. The approvals for natural product derivatives surpassed those for direct natural products from 1966 to 2015. In the period from 1976 to 1985, which saw the highest number of approvals, 52 compounds were sanctioned by the USFDA. Among these, 40 compounds were natural product derivatives, and 12 were direct natural products. There were no similarities between 1976-1985 and 1985-1995 in the drugs that were directly approved by natural products. However, the number of approved drugs containing natural product derivatives increased from 33 to 41 during this period. In the span of 1996-2005, only 7 direct natural product derivatives received approval, and a limited number of natural product derivatives were sanctioned between 2006-2015 (61).

The quantity of direct natural product compounds experienced a peak up to 1965, reaching its highest point between 1956 and 1965. Subsequent to 1965, there was a rise in the identification of new chemical entities derived from natural product derivatives, while the count of direct natural products decreased. This trend underscores the significance of the active core moiety found in naturally isolated products. The maximum average number of natural product derivatives obtained was 4 between 1976 and 1985. Over the last three decades, there has been an average approval of 3.0 to 3.5 natural product derivative drugs by the USFDA (62).

2.3 Nanomedicine based on natural products

Many isolated natural products show strong pharmacological activities including antitumor, anti-inflammatory, antioxidant, and other beneficial effects as proven *in vitro* (63).

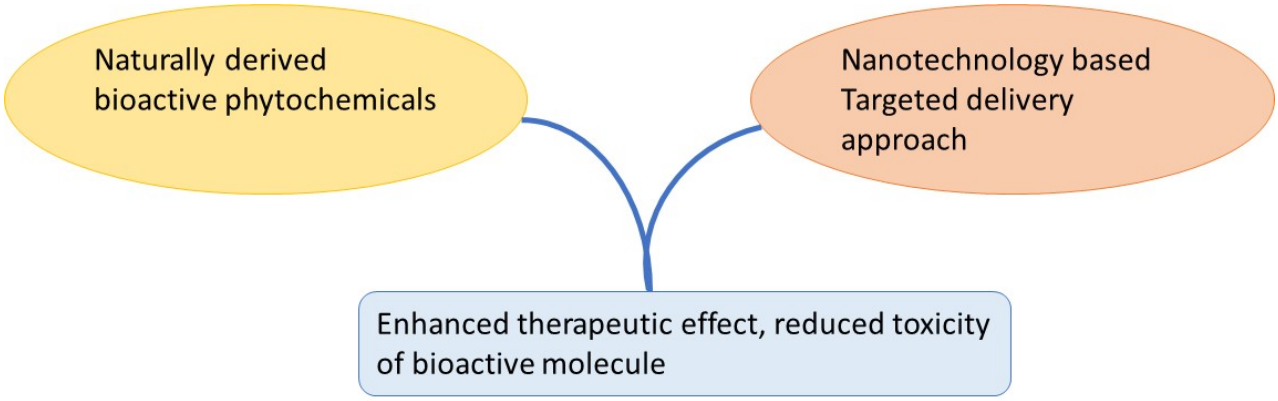


Figure 6 Nanotechnology based approach for delivery of phytochemicals

However, most natural products show limitations such as low hydrophilicity, instability, quick metabolism, low bioavailability, or poor permeability. As a consequence, the *in vivo* effects of these drugs are not ideal and require repeated dose administration beyond the safe range, which is unfavourable in the cases of long-term treatment for chronic diseases (64). Nanotechnology represents a new method to overcome these challenges. The development and application of nanotechnology have greatly promoted the therapeutic efficacy, safety, and patient compliance for drugs derived from natural products. Without a doubt, the combination of natural products and nanotechnology will have great advantages in health care in the future. At present, cancer poses serious challenges to global health. This thesis is based on the application of nano drugs derived from natural products and their use to treat chronic human diseases such as lung cancer (65).

2.4 Oleanolic Acid (OLA)

Oleanolic acid (OLA) is a natural product that has been isolated from several food and medicinal plants (66). It is a pentacyclic triterpenoid which is abundant in plants of the Oleaceae family such as the olive plant (67, 68). In these plants, OLA is often found in the epicuticular waxes where they act as a barrier against pathogens and water loss (69). Apart from its ecological roles in plants, some pharmacological activities such as anti-oxidant, anti-

tumour, anti-inflammatory, anti-diabetic, anti-microbial effects have been attributed to OLA in different models of diseases (70-73).

2.4.1 Physical and Chemical Nature of Oleanolic Acid

Oleanolic acid is a triterpenoid which exists in nature as a free acid or as an aglycone of triterpenoid saponins and it is often ubiquitously found with its isomer, ursolic acid (74). The molecular formula and weight of OLA are $C_{30}H_{48}O_3$ and 456.70 g/mol respectively (75). It has also served as a framework for additional modifications to achieve semi-synthetic OLA derivatives for increased potency, reduced toxicity, increased bioavailability and solubility.

2.4.2 Occurrences of Oleanolic Acid in Food and Medicinal Plants

Pentacyclic triterpenes, including OLA are widespread in the fruits, leaves, and stem bark of various edible and medicinal plants (76). Medicinal plants such as *Lantana camara* (77) and *Lisgustrum lucidum* (78) are rich sources of oleanolic acid and they have been used traditionally for the treatment of various diseases. OLA can easily be obtained in high yield from the olive plant, its main commercial source. Guinda and colleagues reported that plant species, geographical origin, stage of development, and environmental conditions are factors that may influence the level of oleanolic acid in plants. Common culinary spices such as garden thyme and clove plants are also sources of oleanolic acid. Apple, loquat, grape, elderberry, and sage are some of the fruit plants in which oleanolic acid has also been detected and isolated.

2.4.3 Extraction, Isolation and Characterization of Oleanolic Acid

Dry roots of *Lantana camara* were used to extract OLA using the described process with a few alterations (79). Fresh *Lantana camara* roots were carefully collected and thoroughly cleaned to remove any soil or organic matter. The cleaned roots were then chopped into small pieces and dried in a shaded area. Once dried, the roots were ground into a fine powder using a grinder. The resulting powdered *Lantana camara* roots, weighing 800 g, underwent a cold defatting

process using hexane, followed by four consecutive overnight extractions with methanol (MeOH) at room temperature. The extraction process lasted for 8 h until no further extraction was possible, ensuring complete exhaustion of the material. The solvent was then removed under vacuum at 40 ºC, resulting in the formation of a brownish viscous mass, which constituted the crude extract. To further purify the crude extract, it was dissolved in an excess amount of ethyl acetate (EtOAC) and left to stand overnight at room temperature. The resulting mixture was then subjected to filtration and subsequent concentration under reduced pressure at 45 ºC. The obtained concentrated extract was further processed using silica gel column chromatography, employing a series of hexane-ethyl acetate elution solvents with varying ratios (100:0, 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 60:40). This chromatographic separation produced eight major fractions. Among these fractions, fraction 5, eluted with hexane and ethyl acetate in an 80:20 ratio, was selected based on thin-layer chromatography (TLC) analysis. Fraction 5 was subsequently subjected to repetitive column chromatography on a silica gel bed (60–120 mesh size, Merck India) using hexane-ethyl acetate (80:20) as the elution solvent. This purification step successfully yielded pure oleanolic acid with a 1.85 % yield, as confirmed by the presence of a single spot in TLC. Finally, the isolated oleanolic acid was subjected to re-crystallization using methanol (MeOH), resulting in the isolation of pure oleanolic acid.

Apart from OLA, the roots of Lantana camara also contain various mono and sesquiterpenes, triterpenes, and flavonoids. Some of the phytochemicals found in the roots of this plant are ß-sitosterol, lantadene A, ß-sitosterol glucoside, and camaric acid etc.

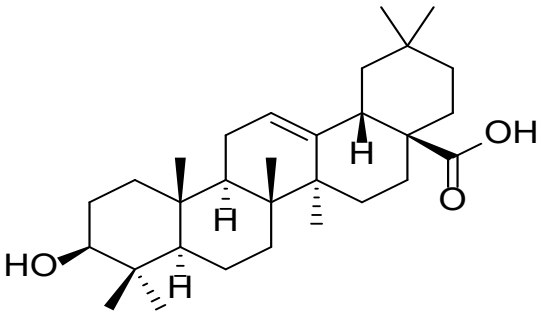


Figure 7 Chemical structure of OLA

Solubility: Chloroform (Very Slightly), DMSO (Slightly, Heated), Methanol (Slightly, Heated).

Molecular weight: 456.3603 Da + Na^+ (22.9892) = 479.3495 (Expected), found: 479.3496

Molecular formula: $C_{30}H_{48}O_3$

2.4.4 Anti-Cancer Effects of Oleanolic Acid

A number of studies have reported the anti-tumour and anti-cancer activities of oleanolic acid against tumour and cancer growth in different *in vitro* and *in viv*o models. For example, OLA inhibits the growth of transplanted tumour in mice and the proliferation of liver hepatocellular cells (HepG2). It was suggested that the anti-tumour activity of OLA is through the upregulation of the tumour protein (p53), cyclooxygenase-2 (COX-2) mediated activation of mitochondrial

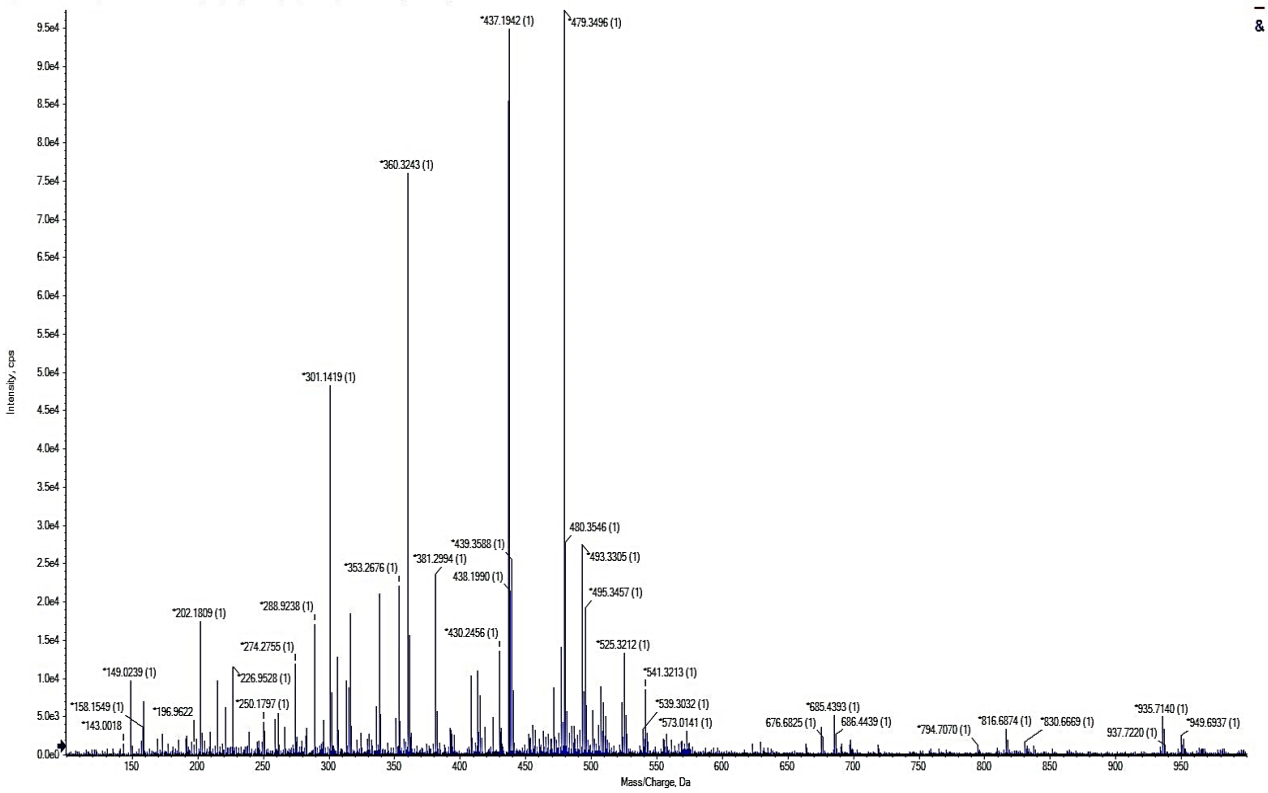


Figure 8 HRMS spectra of isolated oleanolic acid

apoptotic pathway and cell cycle arrest (80). On the other hand, there was an induction of cell death by treatment with a combination of second mitochondrial-derived activator of caspases (SMAC) mimetic BV6 and OA in human hepatocellular cells (81).

SMAC mimetic BV6 is a synthetic selective antagonist of inhibitors of apoptosis (IAP) proteins and hence, also a therapeutic candidate in the treatment of cancer (82). Furthermore, in osmotic stress-induced breast cancer growth, OLA reversed the expression of glycolytic enzymes which were previously enhanced by the hypertonic condition. This reversal efficiently led to a decreased cancer cell proliferation (83). In human bladder cancer cells, treatment with 50 µM of OLA subdued proliferation and enhanced apoptosis of the cells through inhibition of Akt/mTOR/S6K and ERK1/2 (pathways crucial to cell growth, proliferation and survival) signalling (84).

Another specific mechanism of anti-tumour action of OLA that has been suggested is the induction of overexpression of miR-122, a protein which has been found to be an important tumour suppressor in some types of cancer (85, 86). OLA induced the expression of miR-122 in lung cancer cells up to 9.9 folds following treatment with 60 µg/mL of OLA for 8 h (87). In an attempt to enhance the water solubility of OLA, Ren et al. prepared a solid inclusion complex of OLA with amino-appended ß-cyclodextrins. Apart from a considerable increase in solubility, they recorded an enhanced *in vitro* cytotoxicity of the inclusion complex on human cancer cell lines [48]. Generally, these studies suggest that OLA can be valuable therapeutic agent against tumour and cancer through their diverse mechanisms of action.

2.4.5 Problems associated with Oleanolic acid

According to the Biopharmaceutics Classification System, OLA is a class IV drug. Due to its low permeability (Papp = 1.1-1.3×10^{-6} cm/s in the apical to the basolateral direction at 10 and 20 µM), it has an absolute oral bioavailability of only 0.7% and low aqueous solubility (<1

μg/ml) (88). OLA cannot be absorbed completely when taken orally due to its strong hydrophobicity and thus, the therapeutic effects of OLA might be limited. This has restricted the use of various pharmacological activities of OLA for treating different disorders. In recent years many groups of researchers have used different techniques, for example, the preparation of different crystal forms of OLA by recrystallization, preparing solid dispersion, nanoemulsions and nanosuspensions to increase the bioavailability of OLA.

Solidified powder of the OLA-Phospholipid complex was prepared by a feasible and straight forward solvent method to improve the dissolution of OLA and OLAPC. As a result, solidified powder of OLA-PC would be a prospective and practical drug formulation. The hollow MOF-5 (metal-organic framework) was successfully synthesized as a drug delivery vehicle to solve the load-bearing problem of insoluble anti-tumor drug OLA by using the solvothermal method. Zhang et al. developed polymeric nanoparticles. These were developed by vitamin E-modified aliphatic polycarbonate polymer to promote oral absorption of oleanolic acid. Wang et al. developed OLA nanoparticles containing amphiphilic carboxylated cellulose-g-poly (Llactide) copolymer to enhance the drug delivery of OLA. López-Miranda et al. synthesized oleanolic and maslinic acids complexes to improve the bioavailability and extractability of OLA and MA by increasing their aqueous solubility. Recently, Yun et al. studied the metabolic potential of OLA investigating via human P450-catalyzed oxidation reactions. Out of all tested human P450s, only CYP3A4 was active in the hydroxylation of OLA. These results showed that CYP3A4 can hydroxylate an OLA substrate to make 4-epi-hederagenenin. Banarse et al. developed he lyophilized oleanolic acid nanosuspension (OLA-NS) by using the whole whey as one of the stabilizing and bulking agents. OLA-NS was analyzed for various parameters and found to have better dissolution rate and long-term stability. This study could be useful for other poorly water-soluble drugs.

2.5 Asiatic acid (ASA)

2.5.1 Natural sources of Asiatic acid

A monocarboxylic acid called Asiatic acid (ASA; 2α,23-dihydroxyursolic acid) is produced when ursane is hydrided. Ursane is replaced in the structural formula by hydroxyl groups at C-2, C-3, and C-23 (stereoisomer 2α, 3β), as well as a carboxyl group at C-28. The chemical formula of ASA is $C_{30}H_{48}O_5$, and its molecular weight is 488.70 g/mol. According to González-Coloma et al. asiatic acid, like other triterpenoid (TPs), is a secondary metabolite that defends plants from insect and microbial attack. The leaves of *C. Asiatica*, also known as gotu kola or kodavan, have particularly high amounts of ASA both in its free form and as an aglycone with attached carbohydrate residues (asiaticoside). A total of 30% ASA, 40% asiaticoside, and 30% madecassic acid are present in the TPs of *C. asiatica*. South Africa, Australia, Oceania, and Southeast Asian nations (mostly India and China, but also Japan, Malaysia, and Indonesia) are home to this plant (89). Asiatic acid (ASA), a pentacyclic triterpenoid that occurs naturally, is mostly present in the traditional herb *Centella Asiatica*, *Shorea robusta* etc. The main chemical components of *C. Asiatica*, triterpenoid saponins, are thought to be the cause of its vast medicinal effects. *Psidium guajava*, *Combretum fruticosum*, and other organisms with data are known to contain the natural substance asiatic acid (90). The chemical structure of ASA is shown below.

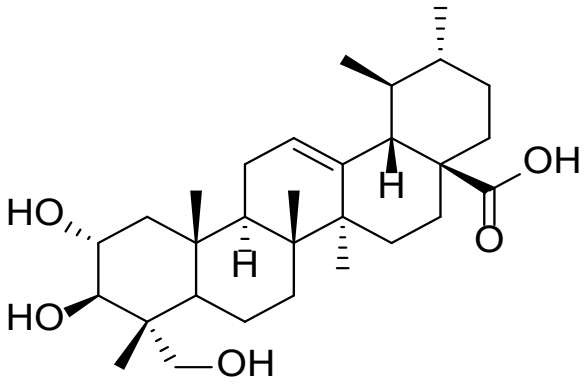


Figure 9 Chemical structure of ASA

- Synonym(s): 2α,23-Dihydroxyursolic acid, Dammarolic acid.
- Solubility: Ethanol: 10 mg/mL, DMF: 20 mg/mL, DMSO: 20 mg/mL, DMSO: PBS (pH 7.2) (1:3): 0.25 mg/mL.
- Molecular weight: 488.3502 Da + Na^+ (22.9892) = 511.3394 (Expected), found: 511.3384
- Melting Point: 318 ºC
- Molecular formula: $C_{30}H_{48}O_5$
- Specific rotation: 50.3 ºC (C=0.25, MeOH)

2.5.2 Extraction, Isolation and Characterization of Asiatic Acid

The dried powdered resin (500 g) of *Shorea robusta* plant was defatted with n-hexane for 24 h. The defatted residue was then extracted at room temperature with methanol (3×5 L) for 48 h each time. The methanolic extract was dried under vacuum at 40°C - 45°C and partitioned between n-butanol and water. The n-butanol part was dried under vacuum to yield 130 g of material that was processed for isolation of the metabolites. The crude residue from the n-butanol extract was subjected to column chromatography over silica gel (60-120 mesh). Graded elution was carried out with chloroform followed by various mixtures of $CHCl_3$-MeOH (98:2, 95:5, 90:10 and 80:20). A total of 60 fractions (250 mL each) were collected, and fractions giving similar spots on thin layer chromatography were combined. Fractions eluted with $CHCl_3$-MeOH (95:5) were combined together and subjected to rechromatography over silica gel to isolate a colourless solid (0.029%). The compound was crystallized from methanol-acetonitrile mixture and characterized as ASA by spectroscopic analysis, viz., HRMS, FTIR and ^1H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy followed by comparison with data reported in the literature (91, 92). Apart from ASA, resin of *Shorea robusta* also contains phytochemicals such as ß-sitosterol, tannic acid and ursolic acid etc.

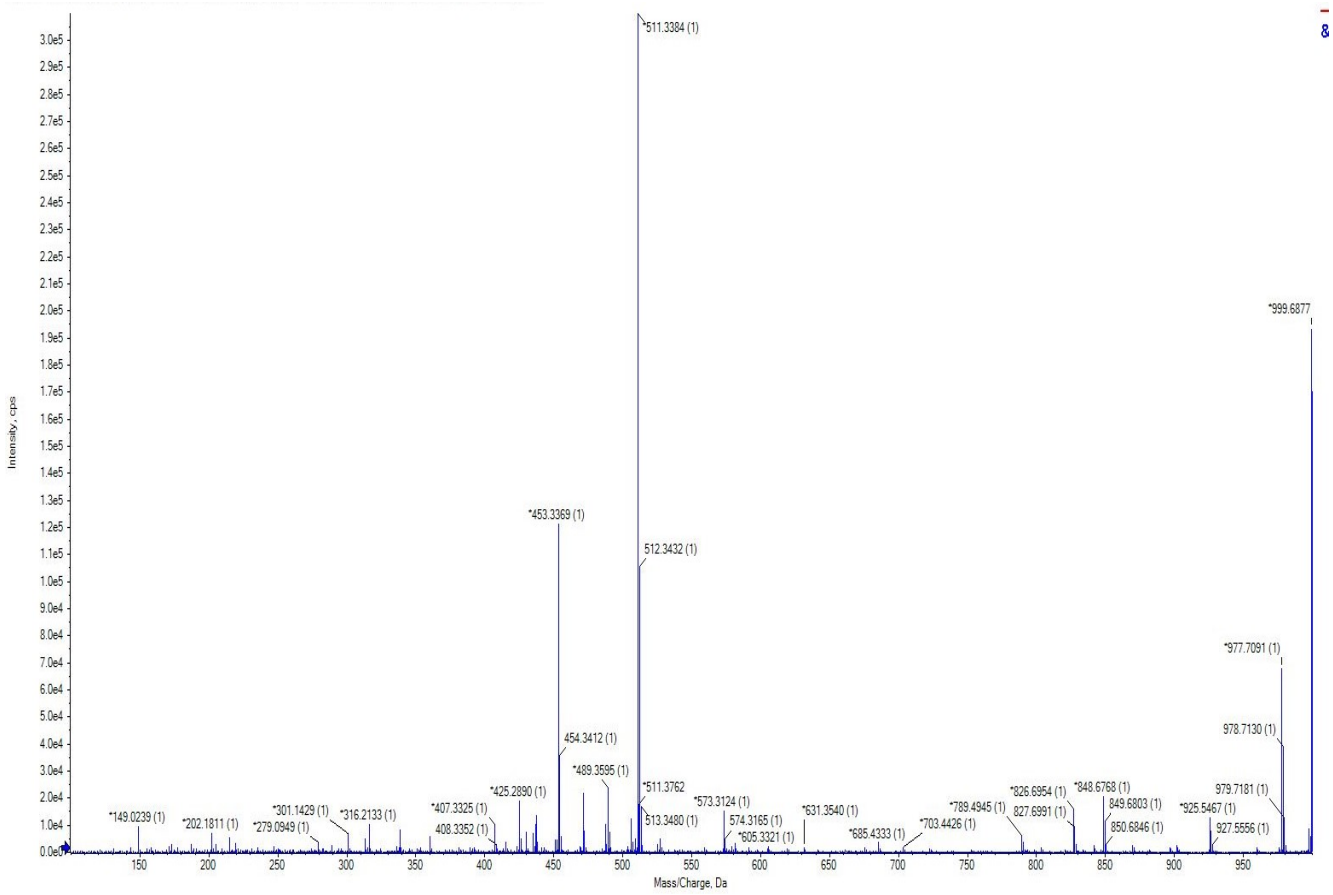


Figure 10 HRMS spectra of isolated asiatic acid

2.5.3 Anti-Cancer Effects of Asiatic Acid

ASA has attracted great interest, and its anticancer effects have been studied *in vitro* and *in vivo* by many researchers in different types of cancer. The interactions of ASA with molecular targets like nuclear factor erythroid-derived 2-like 2 (Nrf2), nuclear factor kappa B (NF-κB), protein kinase C (PKC), free radicals scavenging, and cell longevity pathway (CLP) that lead to anticancer, cytoprotective, chemosensitizer, and chemopreventive effects must be emphasized, as ASA acts by inhibiting the proliferation of cancer cells and induces apoptosis (93). Many studies reported that ASA could induce apoptotic cell death by modulating the protein expression of several apoptosis regulators, such as caspases, B-cell CLL/lymphoma 2 (BCL-2) family members, and glioblastoma multiforme (GBM) cells. BCL-2 and BCL-XL

proteins were decreased by ASA treatment (94). Furthermore, ASA induces endoplasmic reticulum stress (by increasing GRP78 and calpain, and decreasing calnexin and IRE1α expression), enhances free intracellular calcium, and damages cellular organization in GBM cells. Disruption of the endoplasmic reticulum and variations in calcium homeostasis are early events in ASA-induced death. ASA inhibits the expression of NDR1/2 kinase and promotes the stability of the p21WAF1/CIP1 protein through attenuating the NDR1/2-dependent phosphorylation of p21WAF1/CIP1 in Hep G2 cells (95). ASA induces apoptosis through increased intracellular Ca^{+2}, which in turn enhances p53 expression in HepG2 cells (96). Cho et al. also found that ASA induces cell death by both apoptosis and necrosis, with Ca^{+2} mediated necrotic cell death (97). ASA was found to significantly decrease interleukin-8 (IL-8) production in human colon cancer cells. Park et al. also observed that ASA-induced apoptosis may be mediated through the generation of reactive oxygen species (ROS), alteration of the Bax/BCL-2 ratio, and activation of caspase-3, but it is p53 independent (98). Tang et al. illustrated that the mitochondrial death apoptosis cascade plays a very important role in ASA-induced cancer apoptosis. Through the increase in mitochondrial membrane permeability and release of cytochrome C from mitochondria into cytosol, ASA induces caspase-9 activity, which further stimulates caspase-3 and poly(ADP-ribose) polymerase cleavage, resulting in irreversible apoptotic death in tumor cells (99). Taken together, these works suggest that the mitochondrial death apoptosis cascade plays a key role in ASA-induced cancer apoptosis.

ASA significantly upregulates miR-1290, which sensitizes cells to ASA-induced cytotoxicity and negatively regulates BCL-2 expression (100). ASA is able to regulate cell cycle progression in RPMI 8226 cells with the inhibition of signal transduction mediated by focal adhesion kinase (FAK). ASA may exert anti-tumorigenesis through inhibitory actions in NO and cyclooxygenase-2 (COX-2) signals (101). Kavitha et al. also reported the strong antiangiogenic potential of ASA and suggested its usefulness against malignant gliomas (102).

Nuclear factor kappa B (NF-κB) has been known to regulated multiple transcriptional target genes which are associated with innate and adaptive immune responses, tumor processes, and cellular growth and apoptosis. *In vitro* study of A549 cells, asiatic acid significantly suppressed the NF-κB activity, cell migration, and induced apoptotic cell death. In a dose-dependent manner, inhibition of proliferation, induction of cell cycle arrest in G0/G1 phase, blockade of G1-S transition, reduction in cyclin-dependent kinase CKD4, cyclin D1, and phosphorylated retinoblastoma protein levels, enhancement in cyclin-dependent kinase inhibitor P15, suppression of migration and invasion stages, and down-regulation of expression of MMP-2 and MMP-9 were reported in human SGC7901 and HGC27 human gastric cancer cells after asiatic acid treatment. ASA inhibited proliferation, migration and induced apoptosis of colon cancer cells by regulating Pdcd4 via the PI3K/Akt/mTOR/p70S6K signaling pathway (103).

Table 2 *In Vitro* and *In Vivo* anticancer Activities of ASA.

In Vitro anticancer activity	*In Vivo* anticancer activity
↑ Apoptosis in human SK-MEL-2 melanoma cells ↑ Intracellular Ca2+ ↑ Expression of p53 ↑ Extracellular signal-regulated kinase ↑ p38 mitogen protein kinase pathways ↑ Human breast cancer apoptosis ↑ Cell cycle arrest	↓ Tumor volume and weight ↑ Apoptosis of lung cancer cells ↓ DMBA (TPA) ↑ Mouse skin tumorigenesis ↓ NO and COX-2 signaling

2.6 Nanomedicine in the treatment of non-small cell lung cancer (NSCLC)

Currently, nanomedicine-based formulations such as Abraxane and Doxil are available in the market and are being used clinically (104). The nanotechnology-based formulations such as nanoparticles, liposomes, micelles, solid-lipid nanoparticles, etc. can be used by both such drug delivery strategies as active and passive targeting. The extravasation into tumour endothelium is the most prominent pathway for the size-based passive targeting through EPR (enhanced permeability and retention) effect (105). Moreover, the specific types of receptors overexpressed in lung cancer are the key features for the active targeting through the interaction between corresponding receptors and ligand-decorated nanomedicines, followed by the promoted endocytosis with consequent drug release. A huge number of targeting ligands for active targeting in lung cancer have been identified. The nanomedicines, decorated with such targeting ligands, can specifically target lung cancer and improve the therapeutic efficacy of anticancer drugs. The active targeting of the nanomedicines can be facilitated by various targeting ligands such as folate, transferrin, aptamers, monoclonal antibodies etc (106).

Generally, the nanomedicines can be conjugated with targeting ligands either by pre-conjugation or post-conjugation techniques. Personalized nanomedicine requires extensive clinical research on the human body, from which personalized and targeted nanomedicine can be developed. Under the current circumstances, it is challenging to implement personalized nanomedicine for the treatment of a large number of patients. Therefore, the concept of receptor targeted nanomedicine, based on the expression levels of receptors in a large number of patients, is recommended as a promising strategy for lung cancer nanomedicine.

Furthermore, to augment their accumulation in cancer cells, targeted nanomedicine may also be decorated with cell-penetrating peptides such as penetratin, TAT, VP22, polyarginine, etc. The biological response of targeted nanomedicines can vary depending on the density of the

targeting ligands so that for the development of receptor targeted nanomedicine, it is necessary to optimize the proportions of the targeted ligands based on *in vitro* and *in vivo* studies.

Targets for lung cancer	Expression in NSCLC	Nature of receptors/proteins
EGFR	40- 80 %	Transmembrane receptor

2.6.1 Albumin (ALB) as a nanocarrier for cancer therapy

Albumin is a versatile protein used as a carrier system for cancer therapeutics. As a carrier, it can provide tumor specificity, reduce drug-related toxicity, and maintain the therapeutic concentration of therapeutic moiety like drugs, genes, peptides, proteins, etc., for a long period of time, and also reduce drug-related toxicities. It also has the potential in the half life extension of drug. As albumin has various binding sites, ligand-functionalized delivery of therapeutic moiety is also possible, which can provide site-specific delivery of the therapeutic moiety (107). Two basic approaches are utilized in the development of albumin-based cancer therapy systems, i.e., conjugation of therapeutic moiety directly to the albumin or formulation of nanoparticles incorporated with therapeutic moiety like drug, peptide, gene, etc. Some of the biological applications of albumin conjugates are used as a reagent for immunoassay and immunohistochemistry, for elucidating hormone-receptor interactions, and in the treatment of various diseases like cancer, viral infection, and diabetes (107, 108). Albumin-based nanoparticles are utilized for cancer treatment as they are biodegradable, non-antigenic, and can also be surface-modified, which may help in avoiding the undesirable toxicity of drugs by modifying their body distribution and improving their cellular uptake. They also have targeting potential because proteins themselves act as passive as well as an active targeting moiety. Other targeting ligands can also attach to these carriers to provide site specificity [11].

Albumin is a protein-based macromolecule and the most abundant plasma protein (35–50 g/L human serum) of human blood, which is synthesized in the liver at the rate of approximately 0.7 mg/h for every gram of liver (10–15 g daily). It is non-toxic, biodegradable, biocompatible, highly water soluble, non-immunogenic, easy to purify, and stable plasma protein.

Bovine serum albumin (BSA) is obtained from bovine serum and has a molecular weight of 6.93 kDa with pI of 4.7 in water at 25 °C. It is a water-soluble monomeric protein consisting of 583 amino acid residues. It contains 17 disulfide bonds resulting in nine loops formed by the bridges, one cysteine, and 8 pairs of disulfide bonds. It also contains a high content of aspartate (Asp), glutamic acid (Glu), alanine (Ala), luciene (Luc), and lysine (Lys). It is also used as a drug carrier because of its low cost, ease of purification, unusual ligand binding properties, biocompatibility, biodegradability, non-toxicity, lesser immunogenicity (as compared to OVA and rat albumin), and wide acceptance in the pharmaceutical industry.

Albumin-based nanoparticles are widely explored protein-based nanocarriers for cancer therapy. Several categories of drugs can be encapsulated in albumin via covalent conjugation, electrostatic interaction, or hydrophobic interaction, this makes albumin a versatile drug delivery carrier. Apart from this, its accumulation in the tumor also makes it a suitable candidate for cancer therapy.

Methods of preparation of polymeric nanoparticles

a.) Solvent evaporation

In this method, the polymer solutions are prepared in volatile solvents, and emulsions.

b.) Dialysis method

The dialysis method is often used to fabricate small, monodispersed polymeric nanoparticles.

c.) Coacervation/precipitation

The coacervation/precipitation process is based on physical and chemical characteristics of carrier.

d.) Emulsion-droplet coalescence

The emulsion-droplet coalescence approach makes use of both precipitation and emulsion.

e.) Reverse micellization

Reverse micelles are thermodynamically stable and depends on dynamic compositions of water, oil.

f.) Ionotropic gelation

Ionotropic gelation is the process by which a polyelectrolyte is cross-linked with a counter ion.

2.6.2 D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS)

Recently, some non-ionic surfactants with PEG chains such as TPGS and Tween-80 are commonly employed in the drug delivery systems to enhance their stability and half-life in the blood circulation. TPGS is a vitamin-E derivative and approved by the FDA, so it is often used as an excipient in various pharmaceutical preparations. Moreover, TPGS is the inhibitor of the P-glycoprotein efflux pump, making the anticancer drug delivery system more effective. In addition, TPGS can also be modified with appropriate chemical groups such as TPGS-COOH, TPGS-NH_2, TPGS-SH, etc., so that necessary functional groups can be used to bind ligands over nanomedicine surface.

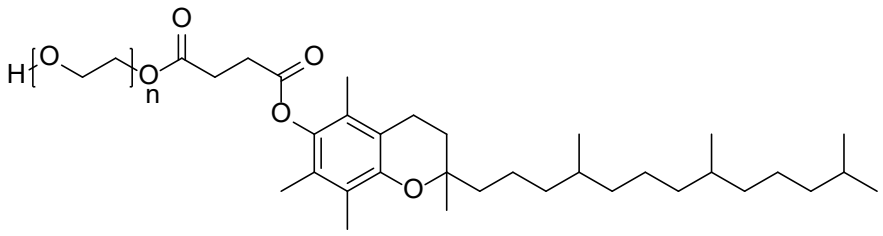


Figure 11 Chemical structure of TPGS

- IUPAC name: [2R-[2R*(4R*,8R*)]]-α-[4-[[3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-2H-1-benzopyran-6-yl]oxy]-1,4-dioxobutyl]-ω-hydroxypoly(oxy-1,2-ethanediyl)

- Synonym(s): D-α-Tocopherol polyethylene glycol succinate, TPGS, Vitamin E polyethylene glycol succinate, Vitamin E-TPGS
- Molecular formula: $C_{33}H_{54}O_5(C_2H_4O)_n$
- Molecular weight: 530.78 + $(44.05)_n$ Daltons
- Solubility: H_2O: 1 g/10 mL, clear to faintly turbid, colorless to faintly yellow

  DMF: 10 mg/mL, DMSO: 5 mg/mL, Ethanol: 15 mg/mL, PBS (pH 7.2): 1 mg/mL.

  Tocofersolan is a polyethylene glycol derivative of α-Tocopherol. Tocofersolan is a synthetic water-soluble vitamin E, unlike its natural counterpart, which are fat soluble.

  Chloroform (Sparingly), Ethyl Acetate (Slightly), Methanol (Slightly, Sonicated)

2.6.3 Preparation methods of albumin nanoparticles

There are different fabrication methods to produce albumin-based nanoparticles. They are classified in chemical-based methods, which use chemical additives, such as ethanol, cottonseed oil, or β-mercaptoethanol, to induce nanoparticle formation, and physical-based methods, which take advantage of physical factors, such as heat or pressure, in order to generate nanoparticles (109). Among the chemical-based techniques, desolvation, emulsification, and self-assembly are the most commonly used methods. Nanospray drying, thermal gelation, and NAB-technology belong to the physical-based techniques. Reproducibility is a key feature that has to be achieved, and every production technique should aim to produce nanoparticles characterized by predictable and reproducible properties.

2.6.3.1 Desolvation (Coacervation)

Desolvation is one of the most used procedures to prepare albumin nanoparticles. It involves the addition of a desolvating agent such as ethanol or acetone in a continuous and dropwise manner to an aqueous solution of albumin under stirring until turbidity of the solution is reached (110). The desolvating agents work by changing the tertiary structure of albumin

gradually, leading to phase separation and aggregation of the protein. In fact, the homogeneous solution separates into two phases, one of which is constituted mainly of solvent and the other of solute, albumin, that forms submicronic aggregates. Most of the time, the obtained formulation is not stabilized enough, and a crosslinker, such as glutaraldehyde, is used to further preserve and stabilize the morphology of the resulting nanoparticles. The properties of the resulting formulation depend on the conditions of the process, such as pH, protein concentration, cross-linker concentration, desolvating agent level, ionic strength, and stirring speed (111).

2.6.3.2 Emulsification

The emulsification process involves the addition of a nonaqueous solution (oil phase) into an albumin solution (water phase), under stirring, generating a crude emulsion (112). The emulsion can be made uniform by homogenization using a high-pressure homogenizer.

After that, there are two possible methods to stabilize the nanoparticles: thermal heating (temp >120 °C) (113) or chemical treatment using a cross-linker, such as glutaraldehyde.

2.6.3.3 Self-Assembly Method

Self-assembly relies on the formation of albumin nanoparticles due to the increase in the hydrophobicity of the protein by breaking of disulfide bonds caused by the use of β-mercaptoethanol or reduction of primary amine groups on the surface of the protein caused by the addition of a lipophilic compound (114). The result is the self-assembly of albumin and the formation of nanoparticles in an aqueous environment.

2.6.3.4 Thermal Gelation

Thermal gelation is characterized by heat-induced protein conformational change and unfolding, followed by protein-protein interactions, such as the formation of hydrogen bonds, electrostatic, hydrophobic interactions, and disulfide-sulfhydryl interchange reactions (115).

The properties of the obtained formulation depend on the conditions of the process, such as pH, protein, concentration, and ionic strength.

2.6.3.5 Nanospray Drying

Nanospray drying is a versatile technique, (116) commonly used to produce a dry powder from a liquid phase. One of the main advantages of this method is that particles are dried and produced in a continuous and single-step process (117). It is characterized by the spray generation of droplets from a liquid solution. The process includes different steps, such as atomization of feed into a spray, spray-air contact, drying of spray, and separation of dried product from the drying air (118). A liquid feedstock is atomized into a spray of droplets and brought into contact with a drying gas, at a sufficient temperature to obtain moisture evaporation. The contact takes place in a drying chamber, where an aqueous solution of albumin is held. As the moisture evaporates, the solid dried particles are formed and collected using an electrostatic particle collector. Optimization of the nanospray drying parameters allows regulating the properties of the nanoparticles, making them suitable for specific applications (119).

2.6.3.6 Microfluidic Mixing

Even though less has been investigated, microfluidic technology is another technique used to produce albumin nanoparticles (120). It provides an effective alternative for the fabrication of lipid, polymeric, and serum albumin nanoparticles. This technique is a controllable preparation process, which results in particles with tunable size and narrow size distribution. Furthermore, it provides a unique opportunity for automatized large-scale, pharmaceutical production. In the literature, there are few studies on the production of albumin nanoparticles under certain conditions. Successful results have been obtained in a recent study conducted in 2020 (126), which was focused on the preparation of core-shell type, drug-loaded albumin-based nanoparticles. The stabilizer poly(allylamine hydrochloride) (PAH) was added to channel 1

(v1) in the first syringe pump, while the solution containing the carrier and the drug (BSA/KYNA) was filled into the channel 2 (v2) in the second syringe pump. After passing through the syringe pumps, the two solutions were mixed in the μ-mixer cell, with a volume of 250 μL, and a pressure controller apparatus. After that, the sample was collected in defined time intervals. A paclitaxel-loaded disulfide-cross-linked (biocompatible alternative to cross-linking with glutaraldehyde) HSA nanoparticles were produced in a microfluidic platform (121). The entire process includes four steps. In the first step, the pretreatment step, HSA was incubated with deionized water with GSH to reduce the 17 disulfide bonds to free sulfhydryl groups. In the second step, the mixing and coprecipitation, the HSA/water solution was mixed with paclitaxel/tertiary butyl alcohol (TBA, an organic solvent used as antisolvent to water) in a microchannel reactor: therefore, there were three liquid inlets and an additional air inlet to form a segmented gas−liquid flow. In the mixing solution, both paclitaxel and albumin were subjected to a huge decrease of solubility, and they precipitated out together, forming PTX-HSA nanoparticles. The third step was the reaction step, where the suspension was incubated at 37 °C to form disulfide bonds. In the final step, dialysis, the suspension was dialyzed against deionized water at 4 °C to remove TBA.

2.6.3.7 Nanoparticle Albumin-Bound (Nab)-Technology

NAB-Technology is one of the most used and popular techniques involving albumin. It is a modified version of the emulsion method described before. NAB-Technology is a nanotechnology-based drug delivery system that takes advantage of the intrinsic properties of albumin to obtain a selective and efficient delivery of hydrophobic drugs without using toxic solvents (122). This breakthrough drug delivery platform allows us to overcome the limitations imposed by the hydrophobic nature of many chemotherapeutic drugs, such as paclitaxel or docetaxel. It was developed to solve the problems that the conventional formulations of these drugs had raised. Taxol (cremophor-ethanol-based paclitaxel (123)) and Taxotere (polysorbate

80-ethanol based docetaxel) have shown acute toxicity, including neuropathy, and hypersensitivity reactions, which are partly due to the use of cremophor and ethanol for the former formulation, and polysorbate 80 and ethanol for the latter (124). It would then be necessary to administer premedication to prevent the deleterious side effects, and as a consequence, the maximum tolerated dose of the drug is low. Another reason why nanoparticle formulations are superior to the alternatives is that solubilizing agents such as cremophor and ethanol can leach plasticizers from PVC bags, which are the commonly used infusion systems. The NAB-technology process is based on an emulsion-based method described before. As mentioned, an oil phase (containing the drug) is added dropwise to an aqueous phase (containing HSA/BSA, presaturated with 1% chloroform), and the mixture is subjected to mild homogenization at low rpm to form a crude emulsion. The final emulsion is obtained by using a high pressure homogenization, and after transferring the mixture in a rotary evaporator, the solvent is removed, and the nanosuspension is produced. It is translucent, and the drug loaded albumin nanoparticles have a diameter of hundreds of nm (generally <200 nm). A 0.22 μm filter is used to control the size of the nanoparticles and to sterilize the formulation, filtering out the impurity and bacteria. After that, the nanoparticles are lyophilized (without adding any cryoprotectant) to obtain solid powders. The original dispersion can be recreated by the addition of water or saline to the solid nanoparticles.

2.6.3.8 Nab-Paclitaxel (Abraxane)

Paclitaxel is an antimitotic drug that belongs to the family of Taxanes, which are microtubule stabilizers. They bind to the β-tubulin chain and enhance polymerization, inhibiting mitosis, motility, and intracellular dynamics, resulting in cell death (apoptosis).

The paclitaxel formula is $C_{47}H_{51}NO_{14}$, and its molecular weight is 853.91 g/mol.86 It is hydrophobic, and its melting temperature is 216-217 ºC. Nab-paclitaxel (Abraxane for injectable suspension, ABI-007 manufactured by Abraxis Bioscience) is a formulation

constituted of paclitaxel-loaded albumin-based nanoparticles obtained through NAB technology (125). This technology was FDA-approved in 2005, and it has been commercialized to increase efficiency and targeting while reducing the side effects due to the solubilizing agents in the conventional formulation of paclitaxel, as previously discussed. It is currently used for the treatment of metastatic breast cancer, non-small cell lung cancer, metastatic adenocarcinoma of the pancreas (in this case, a combination of Nabpaclitaxel with gemcitabine, an antimetabolite, shows optimal results), bladder cancer, and gastric cancer (in Japan). The formulation is composed of three-dimensional nanoparticles (size of approximately 130 nm) of paclitaxel encapsulated in an amorphous state in HSA through noncovalent hydrophobic interactions. It is free of any toxic solvents/surfactants, and its ζ potential is -31 mV, which shows stability in the aqueous phase.

2.7 Targeted delivery of natural compounds for lung cancer therapy

The main drawbacks of current chemotherapy for lung cancer treatment include a lack of target specificity, recurrence, and a superficial increase in human survival. Furthermore, oral and IV administrations of anticancer drugs have numerous drawbacks, including drug molecule degradation in the stomach pH, drug molecule alterations during the process of metabolism in the liver, and lack of specificity, which causes toxicity and side effects. Poor overall survival rates in NSCLC patients may also be related to inherent radiation resistance due to cancer cells' improved capacity to repair DNA damage after radiation therapy (RT). As a result, it is critical to design a system that can overcome the constraints mentioned earlier and deliver the anticancer drug at the cancer site in the desired concentration.

Targeted therapy is a type of cancer treatment that targets specific genes, proteins, or the tissue environment that promotes cancer development and survival. These medicines are highly targeted and operate differently than chemotherapy. This therapy inhibits cancer cell growth and spreads while causing minimal damage to healthy cells. Currently, the most important

targeted treatment strategies in lung cancer are EGFR inhibitors, VEGFR inhibitors, 4-Anaplastic lymphoma kinase fusion gene (ALK) inhibitors, and B-Raf enzyme (BRAF) inhibitors etc (126).

2.7.1 EGFR inhibitors

The epidermal growth factor receptor (EGFR) is a cell-surface receptor that belongs to the ErbB family of tyrosine kinases and regulates cell proliferation, survival, and differentiation (127). Indeed, EGFR is overexpressed in a wide range of human malignancies, including lung, head and neck, colon, pancreatic, breast, ovary, bladder, and kidney cancers, as well as gliomas. EGFR overexpression is found in more than 60% of non-small cell lung cancers (NSCLCs), but not in small cell lung cancer (128). EGFR overexpression is thought to be generated by a combination of epigenetic processes, gene amplification, and oncogenic viruses. EGFR inhibitors are drugs that disrupt the EGFR signal that instructs cells to grow. Some of these drugs may be used to treat NSCLC.

2.7.1.1 Cetuximab (CTX)

It is a kind of chimeric (mouse/human) monoclonal G1 immunoglobulin that targets and binds to the EGFR's outer domain. In February 2004, the US FDA authorized it for clinical use in treating different malignancies by i.v. infusion (129). In humans, the half-life of cetuximab is around seven days, allowing for once-weekly treatment in conjunction with typical chemotherapy regimens. Cetuximab internalization causes receptor degradation without phosphorylation or activation, decreasing EGFR-dependent downstream signalling cascades. Cetuximab-mediated receptor downregulation causes cytotoxicity by inhibiting receptor function.

Table 3 Cetuximab conjugated nanoformulation for targeted cancer therapy

Targeting ligand	Disease	Delivery system	Outcomes	Ref
Cetuximab	Cancer	Iron oxide nanoparticles	EGFR targeted delivery for cancer theranostic application	(130)
Cetuximab	Colon cancer	Magneto-fluorescent silica nanoparticles	Designed for EGFR-expressing colon cancer targeted theranostic application	(131)
Cetuximab	Lung cancer	Gold nanoparticles radiolabelled with In-111	EGFR expressing A549 tumor-bearing nude targeted theranostic application	(132)
Cetuximab	EGFR overexpressing cancer cells	O-carboxymethyl chitosan nanoparticles	Targeted delivery of paclitaxel to EGFR expressing cancer cells	(133)
Cetuximab	Glioblastoma	Iron-oxide nanoparticles	EGFR targeted glioma theranostic application	(134)
Cetuximab	Colorectal cancer	Citrus pectin-chitosan nanoparticles	EGFR targeted delivery of curcumin for colorectal cancer therapy	(135)
Cetuximab	Non-small cell lung cancer	PLGA nanoparticles	EGFR targeted delivery of docetaxel to the non-small cell lung cancer	(136)
Cetuximab	Breast cancer	Immunoliposomes	For overcoming the multidrug resistance in breast cancer cells	(137)
Cetuximab	EGFR expressing cancer cells	poly (lactic-co-glycolic acid) nanoparticles	Targeted delivery of temozolomide to cancer cells	(138)

2.8 Semi-synthetic modifications of natural products

2.8.1 Click chemistry-inspired structural modification of natural products

The exploration of novel drugs through altering the structure of natural products (NPs) has often faced challenges due to complex and lengthy synthetic routes. Barry K. Sharpless introduced the concept of "click chemistry", offering an effective method to selectively modify intricate NPr structures under gentle conditions, even when reactive functional groups are

present. The copper(I)-catalyzed alkyne-azide [3 + 2] cycloaddition (CuAAC) reaction, particularly, is of interest as it yields 1,4-disubstituted 1,2,3-triazoles (139).

The 1,2,3-triazole group serves as a crucial pharmacophore, showcasing a broad spectrum of pharmacological effects. Its presence holds significance in medicinal chemistry as it facilitates hydrogen bonding, thereby enhancing solubility and enabling favorable interactions with biological targets. Additionally, 1,2,3-triazoles exhibit notable stability against metabolic breakdown due to the presence of three adjacent nitrogen atoms. Numerous 1,2,3-triazole compounds have been studied to date, revealing diverse biological activities.

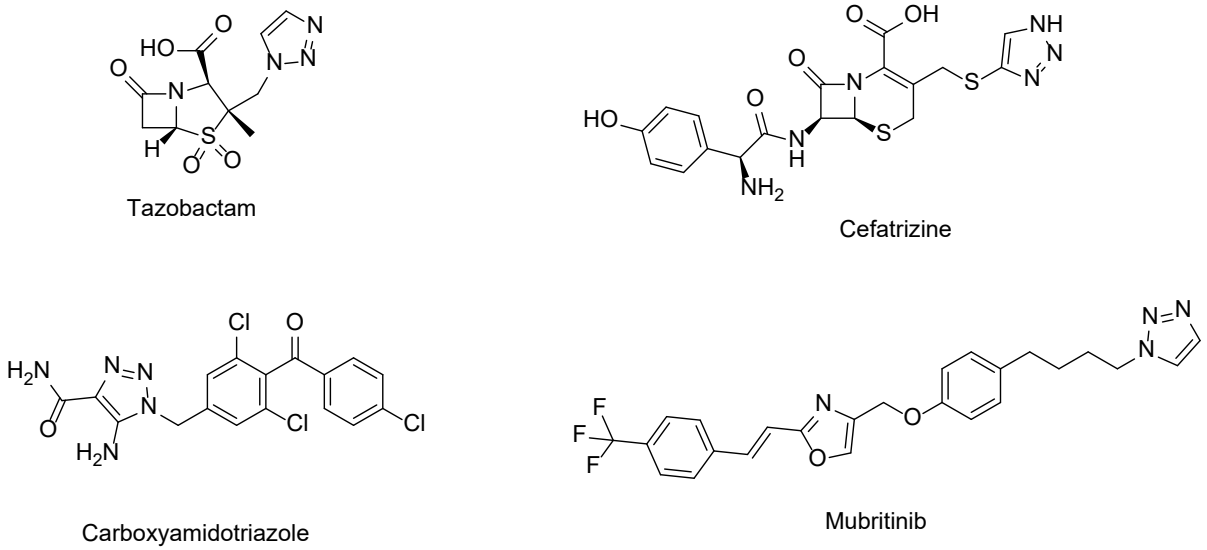


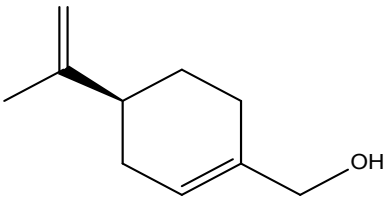
Fig. 12 Biologically active 1,2,3-triazole

Numerous natural product frameworks, including oleanolic acid, quinolone, isatin, myrrhanone C, podophyllotoxin, artemisinin, coumarin, and curcumin, linked with 1,2,3-triazole and possessing hydrophobic traits, have exhibited promising anti-proliferative effects against different cancer types (140). Incorporating the 1,2,3-triazole moiety through conjugation has proven to be a pivotal tactic in enhancing the anticancer attributes of natural scaffolds. This approach has led to the development of numerous secondary leads with potential therapeutic applications.

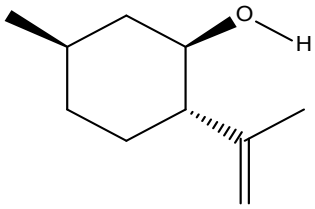
Furthermore, certain compounds containing 1,2,3-triazole, such as Cefatrizine and Carboxyamidotriazole, have either been utilized in clinical settings or are currently undergoing clinical assessment for cancer therapy, indicating their promise as potential anticancer medications (141). This widely recognized pharmacophore exhibits considerable resistance to metabolic breakdown and can engage in dipole-dipole interactions and hydrogen bonding, offering additional benefits such as enhanced cell permeability and binding to targets.

2.8.2 Semi-synthetic modifications of terpenes

Interesting biologically active substances are synthesized from a variety of compounds isolated from natural sources. Structural modifications on these pure monoterpenes were then carried out with a view to enhancing the biological activity. Starting from monoterpenes bearing an alcohol function, a terminal alkyne moiety was introduced, leading to the corresponding O-tethered alkyne. An alkyne was added to the monoterpenes to obtain enynes from which a large variety of structures are accessible (cyclopropane, cyclopentenone, or diene) through metal-catalyzed reactions. These alkynes were then involved in metal-catalyzed click reactions leading to a triazole moiety.



(a) p-Mentha-1,8-diene-7-ol



(b) (1R,3R,4S)-p-Menth-8-en-3-ol

p-Mentha-1,8-diene-7-ol (MDL or PLA), depicted in Figure 13a, is a monoterpene compound obtained from the essential oils of citronella, lavandin, peppermint, spearmint, celery seeds, and several other plant sources. It is a hydroxylated monoterpene synthesized via the mevalonate pathway and serves as the active metabolite of limonene. MDL exhibits dual roles as both a chemotherapeutic agent and a chemopreventive agent against various types of cancers

(142). Its effectiveness has been extensively studied in malignancies such as breast, pancreatic, and brain tumors, where it functions by inhibiting the overexpression of iNOS/NF-κB, in addition to possessing notable anti-inflammatory and antioxidant properties (143).

The p-Menth-8-en-3-ol (IPG), depicted in Figure 13b, is a monocyclic monoterpene alcohol found in the essential oils of numerous plant species, including *Corymbia citriodora* H., *Zanthoxylum schinifolium*, *Melissa officinalis* L., and *Eucalyptus citriodora* H, among others (144). IPG demonstrates the ability to induce apoptosis in HepG2 human hepatoma cells and exhibits potential for preventing various other types of cancers (145).

In summary, 1,2,3-triazole pharmacophores added to a monoterpene skeleton increased the *in vitro* anticancer activity and thus should be further studied through the preparation and evaluation of a larger family of new terpenoids based on these structural features. New monoterpene derivatives could be the basis of a new class of anticancer compounds.