CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Computational Evaluation of *Azadirachta indica* as a Anti-inflammatory and Apoptotic agent for Cancer

by

Maria Khadija

A thesis submitted in partial fulfillment for the degree of Master of Science

in the

Faculty of Health and Life Sciences
Department of Bioinformatics and Biosciences

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I would like to dedicate this to my parents and teachers

CERTIFICATE OF APPROVAL

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Abstract

Azadirachta indica (Neem) is a wonder tree due to its therapeutic, agricultural, domestic, and ethnomedical significance. Pre-clinal work during the last decade has turned the understanding about the anti-cancerous products from this plant. The anti-cancerous property of *A. indica* plant has provided in terms of apoptotic, protective, tumor-suppressive, preventive, and immuno-modularly effects on various types of cancers. Therefore, understanding the molecular mechanism to determine the abnormality is very important as cancerous cells adopt multiple pathways for their survival, and blocking of few pathways does not ensure their target elimination. The text mining technique would be used to find the cancer-relevant pathways affected by *A. indica* compounds and subsequently modeling the interactions of identified compounds along with drug likeliness properties. Consequently, the COREMINE tool was initially used to identify the anti-cancer pathways targeted by *A.indica* compounds. The hybrid Petri net was modeled by using the Snoopy tool to find the cumulative effect of *A. indica* active compounds (Azadirone, Nimbolide, Nimbidin, Azadirachtin, Gedunin) along with a molecular docking-based approach by using MOE to assist the bonding potential of *A. indica* compounds that possess anti-cancer activity. Furthermore, bioavailability and toxicity prediction of compounds had been studied by using SWISSADME. The results from text mining suggested that apoptosis and NFKb inflammatory pathways are significantly targeted by 5 *A. indica* compounds. The Petri Net provided that nimbolide, azadirachtin, azardirone, and gedunin have more significant results for regulating apoptotic pathways. Among five compounds nimbidin targets PEG2 and iNOS production that plays a significant role in controlling macrophages (inflammatory response) by the rate of 0.3 and 0.67 for apoptosis. However, identified compounds Azadirone, Nimbolide, Nimbidin, Azadirachtin, Gedunin have good cumulative effects that if 4 from 0 for apoptosis. The docking results show that Azadirachtin provided good binding interaction with PCNA and P21 proteins by providing maximum binding sites. The Azardirone provides the best interaction with SURVIVIN and XIAP, Nimbidin with ILIB and PEG2 and Nimbolide provides the best interaction with PTGS2 and VEGF. So current

studies provide computational background about *A. indica* compounds involved in regulating the anti-cancerous activity by providing suitable target proteins. This facilitates the screening of novel resources from the combination of identified compounds for combination therapy of cancer.

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Abbreviations

ADMET	Absorption Distribution Metabolism Excretion and toxicity
ASD	Autism Spectrum Disorder
APAF1	Apoptotic protease activating factor 1
DR	Death Receptor
IL1	Inter Leukin 1
iNOS,mRNA	Inducible Nitric oxide Synthase mRNA
MOE	Molecular Operating Environment
NO	Nitric Oxide
PDB	Protein Data Bank
PGE2	Prosta Glandin E2
RMSD	Root Mean Square Deviation
TNF	Tumor Necrosis Factor
VEGF	Vascular Endothelial Growth Factor

Symbols

ft	Feet
^oC	Degree Celcius
1‘	One Prime
2‘	Two Prime
μ	Mu
β	Beta
α	Alpha
κ	Kappa

Chapter 1

Introduction

The medicinal value of the plants in disease management is studied due to affordability and lesser side effects. Natural or plant products show a significant role in disease prevention and cure through inhibition of bacterial growth, enhancement of antioxidant activity, and modulating genetic pathways. The allopathic drugs are costly and may produce toxic effects through different signaling pathways in normal cells. For this reason, numerous pharmacological active medicines are derived from naturally occurring resources [1, 2].

The role of plants in disease prevention and control is defended in the Holy books like in Quran and Bible. According to Islamic history, herbs play a significant role in disease management and our Prophet (P.B.U.H) also recommended various fruits and plants in the prevention and cure of many ailments [3]. Earlier investigators confirmed the role of neem constituents as antiarthritic, anti-inflammatory antipyretic, antibacterial, antifungal, antitumor, hypoglycemic, and anti-gastric ulcer [4–7]. It is estimated that 80% of developing countries used these constituents for alternative medicine for primary care [8, 9]. The migration of people toward developed countries, not only brings their skills but their traditional values. Therefore, the countries like Pakistan, India, and many other such countries, there is the trend of using complementary along with allopathic medicine for energy, balance, or spiritual healing process through several healing benefits such as Sowa-Rigpa and Ayurveda. Especially these traditions continue to begin by using

several therapies through the combination of plants like Turmeric, Neem, Amla, Guggul, and Tulsi [9].

According to ancient Sanskrit, neem is referred to as "Nimba" which particularly expresses a state. The WHO (World Health Organization) defined "good health" as "a state of physical and mental well-being not altered by any disease or ailment" [10]. These days neem is referred to as Azadirachta indica, as it brings "good health" to those who take them [10–12].

Neem is a member of the family called Meliaceae which is abundantly present in tropical and semitropical countries such as Nepal, Pakistan, Bangladesh, and India. Neem shows fast growth and its tree is 20-23 m tall and has a diameter of around 4-5 ft and a straight trunk. The leaves are imparipinnate, compounds having leaflets ranging from 5 to 15. It bears green drupes as fruits which in June and August turn yellow [13].

Neem has various therapeutic implications in Nepal, Bangladesh, Pakistan, and India for disease cure. Neem compounds are famous for their use in curing cancers, infectious as well as metabolic diseases [14]. In many countries, various types of plants or their derivatives play a significant role in disease management. In traditional medicine, the neem tree has been used for many ages as it was mostly grown in southern parts of Africa and Asia to cure multiple chronic and acute diseases [4].

In addition to its therapeutic properties, neem had demonstrated oviposition deterrent, antifeedant and toxic effect on larvae and eggs of armyworm [14–16]. Neem trees, it turns out, are a perfect alternative to modern teeth care products. Also, the leaves of the neem tree were used as a natural acne remedy [17]. Similarly, the use of neem leaves may be used to treat infected eyes. A similar infusion can be used to treat sore throats as well [18]. Neem trees were used to treat several illnesses, including ulcers in the stomach, jaundice, and a variety of parasitic diseases such as malaria, chickenpox, etc., and other viral diseases. It was also used as a diuretic and for diabetes, as well as to treat various skin diseases [19–23]. The extensive study of neem compounds provides the biological effects of plants and

herbs that belong to these families: catechins, limonoids, quercetins, flavonoids, anthocyanins, tannins, garlic acid with some polyphenols all have biological effects as shown in Fig 1.1 [21, 24, 25].

The different parts of this tree like fruit, flower, oil, gums, bark and leaves were used in folklore medical treatment of certain conditions like diabetics, hypertension, and cancer. These extracts are attributed to molecular and cellular mechanisms that include detoxification, free radical scavenging, cell cycle arrest, anti-inflammatory, anti-metastatic, anti-angiogenic, immune surveillance, autophagy and programmed cell death, DNA repair, and can modulate various signaling pathways [4, 5, 10].

Neem is rich in secondary metabolites including non-isoprenoids (sulfur compounds, carbohydrate, dihydrochalcones polyphenolics, and glycosides) isoprenoids (protomeliacins, Nimbin, limonoids, gedunin, azadirachtin, azadirone). The foremost polyphenolic flavonoids that were extracted from the neem fresh leaves were B-sitosterol and Quercetin that showed anti-bacterial as well as anti-fungal activities [7, 10, 26].

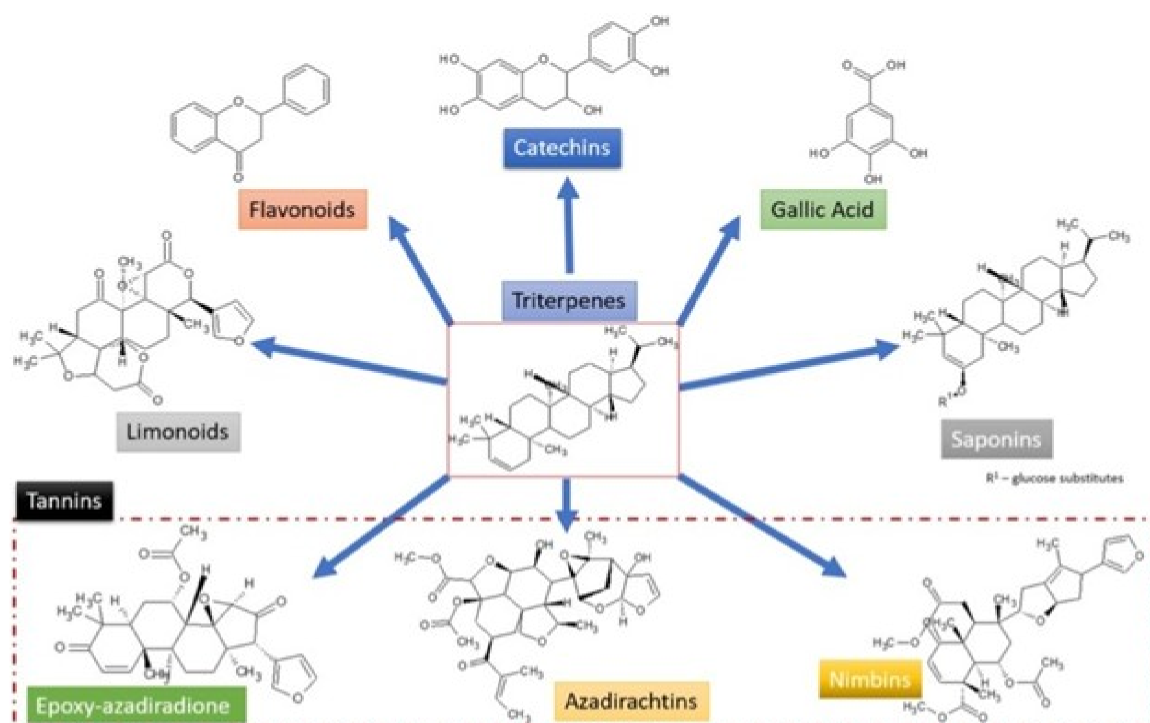

FIGURE 1.1: Effects of neem compounds on various cellular mechanisms [14][6, 26]

The currently available advanced technologies now analyze and investigate dysregulation occurring within transcriptomics, miRNA, genomics, metabolomics, proteomic. However, the major drawback is that in this scientific era, only individually targeted separated molecular levels are explored for influencing health conditions [27].

The employment of computational and modeling science is an important component of system medicine. The computational expertise is a prerequisite for the interpretation of wet lab and effective handling of big datasets such as the formation of complex interactions among molecules. In current years, the earliest human condition that is checked at the system level is Neuroblastoma (NB) [28]. MYCN is a regulatory network model for neuroblastoma oncogene, that gave a deep understanding of the response of tumors by evaluating the impact of retinoid drugs through perturbation of computational model by identification of molecular interaction [29]. The application of humans to preclinical testing in cell lines and animals has changed to the drug screening and testing process. Incorporating these studies with genomics, metabolomics, and proteomics data helps in the rapid screening of medicinal plants [29].

Pathways outside the biological systems have been investigated using an interdisciplinary field known as system biology or computational biology that focuses on bodily symptoms of disease at molecular level in the regulatory network by mathematical modeling of the complex system at the cell, tissue, organ and organism level [30]. The computational and bioinformatics tool-related proteomic, gene expression, metabolomic data are employed to relate the behavior of the cell in disease condition after therapeutic intervention. The system biology provides the profiling and understanding of therapeutic and pharmacological responses based on knowledge of disease components in the treatment system [31]. In certain cases, it can identify the exact molecular mechanism in a particular disease in the patient [32]. Current improvements in modeling and simulation help to predict the response of therapeutic intervention in response to the pathophysiology of various diseases. Various modules provided the pharmacological response of chemical agents on the level of tissue, organ, and cell. To evaluate the anti-inflammatory

properties of compounds, the system biology approach is based on modeling cell-to-cell communication through the construction of neural networks involved in the process of inflammation [33].

The different genes that are part of the anticancer process are computationally constructed. For therapeutic purposes, different forms of plant decoctions and extracts provided the basis of traditional medicine. Most of the research is focused to address the need in research of the effect of multiple compounds on a cellular level and molecular level in response to compound that fits in various molecular pathways that perturbed various genes. This will help in decoding the mechanism of action of drugs. The system biology approach endorses the hypothesis-based biological process and uses particular physiological markers for the authentication of the target in a cost-effective drug discovery process [27].

Petri net approach is one of the computational methods that has been used to construct and analyze the behaviors that represent structural and dynamic properties. Further, the docking study provides the binding affinity of prioritized compounds with genes that are involved in anti-cancerous pathways [34]. The current study has been planned to investigate the bioavailability of Phyto-compounds of neem along with potential targets that manifest multiple signaling pathways in cancerous cells with the help of modeling and docking [30].

1.1 Problem Statement

Azadirachta indica (neem) and its constituents have therapeutic significance that has been traditionally used worldwide especially in the subcontinent since ancient times. The clinical studies provided that *A. indica* plays a central role in the prevention of several diseases. *A. indica* natural extracts have been shown to work as a supplement to control several alignments such as inflammation and diabetics. The role of *A.indica* active constituents with chemopreventive effect has been noticed in several tumors by modulating various signaling pathways. The exact molecular mechanism of *A. indica* extracts in deactivating the activity of several

genes in cancer progression and development in this regard is not understood completely and yet needs to be explored.

1.2 Aims and Objectives

The study focused on modeling and analyzing *A .indica* compounds that are involved in anticancer activity.

1. To identify *A. indica* compounds with therapeutic potential
2. To construct a Petri net model of normal and disease pathways that would be involved in anti-cancerous activity
3. To perform formal modeling for evaluating the cumulative effect of prioritized compounds of *A. indica*
4. To validate the interaction of prioritizing compounds obtained from formal by using the docking technique

Chapter 2

Literature Review

Azadirachta indica, also known as Neem, was a perennial tree mostly used in Ayurveda for centuries [6, 35]. Melia Azadirachta (L) and Antelaea Azadirachta were the previous names for the neem plant (L). Myanmar (Burma) and India were their native countries. It was also found in Sri Lanka, Bangladesh, and African countries. *A. indica* was 20 ft tall that belong to the Meliaceae family. A. India had different names Azadirachta (Persian), Nimba (Sanskrit and Marathi), Dogon Yaro (some Nigerian languages), Kohomba (Sinhala), Azadirachta (Persian), Tamar (Burmese), neem (Hindi and Bangla), Indian lilac (English). It was also referred to as Mwarobaini (Swahili) which is a "tree of 40" in the regions of East Africa due to its use in the treatment of forty diseases [36].

2.1 Taxonomy, Tree Distribution, and Growth

The neem belongs to the genus Azadirachta (neem) and the Meliaceae family the table 2.1 provided the complete taxonomical information. It's a multipurpose tropical evergreen tree native to Indonesia, Thailand, Malaysia, Australia, Burma, Bangladesh, Pakistan [19], [37–39]. *A. Indica* showed growth in a variety of climatic conditions up to 700 meters in altitude and has a lifespan of over 200 years. It thrives in climates with rainfall(50-1, 200 mm), temperatures(0-49 ^{o}C) and pH (4-10). This tree can, however, be cultivated in regions with very low yearly rainfall, such as those with 150–200 mm. The production of its seeds, oil, and cake in India is 442,300, 88,400, and 353,800 tones respectively [40].

Table 2.1: Taxonomic position of A.indica plant

Classification Levels	**Neem**
Order	Rutales
Suborder	Rutinae
Family	Meliaceae
Subfamily	Melioideae
Tribe	Melieae
Genus	Azadirachta
Species	Indica

The neem has light green simple imparipinnate leaves up to 15 narrow and 6cm long leaflets that are alternately arranged in pairs [31, 41] with up to 15 cm narrow and 6 cm long leaflets. The flowers are small, white, or pale yellow, pentamerous and bisexual that was visible from May to August [42]. The plants produce one or two-seeded ellipsoidal drupes that are 1-2 cm in length and are greenish yellow or purple. After ripening they appear greenish, and their seeds are ovoid or spherical when they're four years.The trunk of the neem is straight, with widespread branching and heavy bark. The mature tree reaches a maximum height and girth of 7-15 m and 3 m, respectively [41, 42].

2.2 Phyto-Compounds

The alkaloids, terpenoids, cyclic tri-sulfide, saponins, unsaturated steroid, and flavonoids were the abundant *A. indica* phyto-compounds [43]. The neem leaves possess two recognized constituents isomeldenin and flavonoids and two new compounds zafaral and nimocinol which were tera-cyclic tri-terpenoids. Flavonoids (genistein 7-oglucoside and (-) epicatechin, nimonol, tetranor-tripenoid were further extracted from leaves [44]. In neem tree sitosterol, ferulic acid, quercetnin, ellagic acid and rulitin were found through thin layer chromatography of phyto-constituents. The bark and leaves extract of neem contained terpinene, vitamin E, and nimbol as phyto-compounds [45]. The neem leaves by using ethanol extraction further provided non-terpenoidal components were isolated like nimbothalin and tridecyl benzene. Isoprenoids, flavanone (8,3′ diisopreny l5,7dihydroxy

4′ methoxyflavanone), meliacin (2′,3′ dehydrosalannol) and nonisoprenoids were all included in the neem leaves. From a methanolic extract of leaves of neem, 22, 23dihydronimocinol, triterpenoids, and desfurano6 hydroxyazadiradione were extracted Azadiradione, tetraterpenoid (14, 15epoxynimonol), and flavonoids, especially quercetin, were also found in the leaves. The phytochemicals extracted from the neem tree [45] are shown in Table 2.3

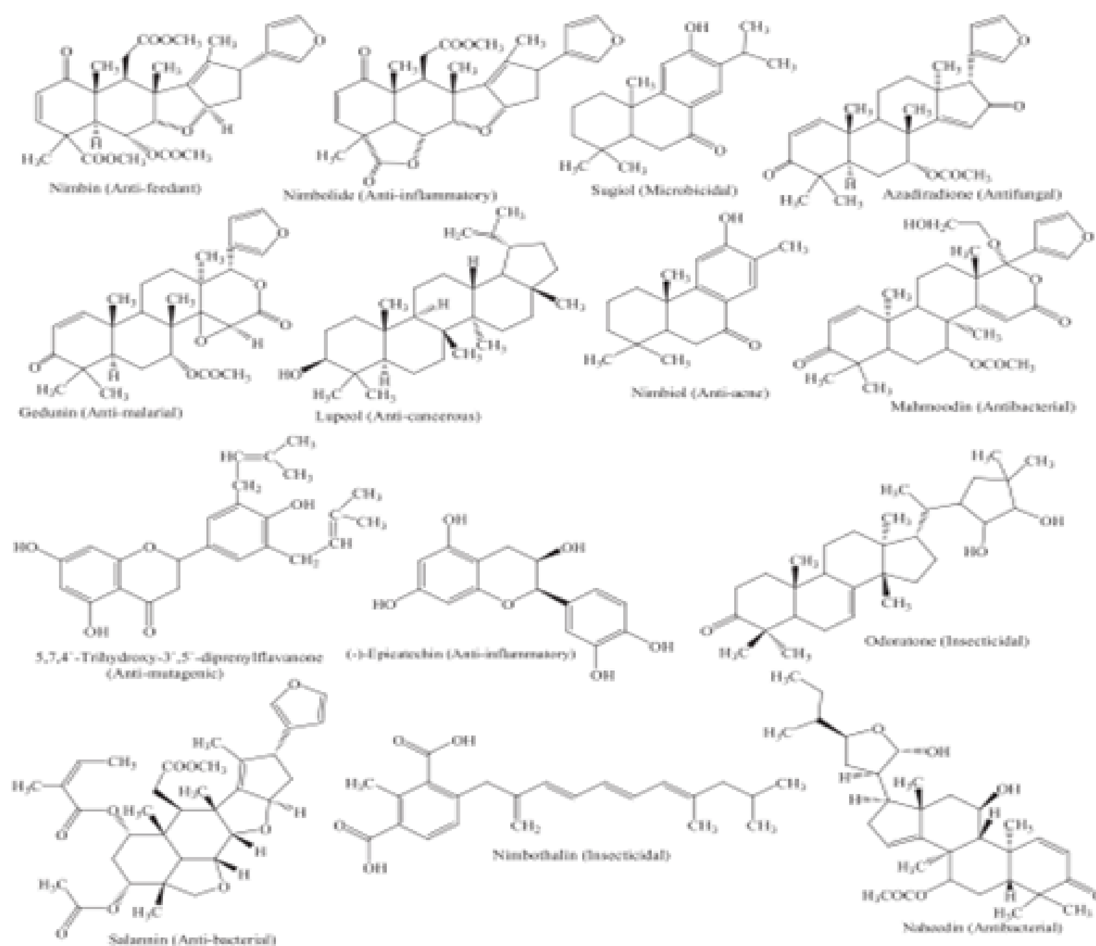

Figure 2.1: The structure of selected biologically active phyto-compounds from *A. Indica* [46]

Azadirachtin and related limonoids were found in the leaves of A.indica, which were harmless to to most of the human beings, and animals. Isonimbinolide and isosalanninolide were generated by photooxidation of nimbin, salannin, and azadirachtin. 2D nuclear magnetic resonance (NMR) indicated the existence of tetraanortriterpenoid, meliatetraolenone, and odoratone in the sample. The 2D nuclear magnetic resonance (NMR) was used to confirm the existence of tetraanortriterpenoid, meliatetraolenone, and a compound called odoratone in fresh

leaves. Hydro distillation was used to extract essential oils from neem leaves and flowers, which were then analyzed by gas chromatography-mass spectrometry (GC/MS). The existence of 85.6 percent hydrocarbons was discovered by GC/MS, including elemene (9.89 percent), caryophyllene (6.8%), eleme (33.39 percent), germacrene D (9.72 percent), and bicyclogermacrene (5.23 percent) [45].

The oil isolated from flowers of neem consisted of 63.22 percent hydrocarbons (pentosane 18.58 percent, tetracosane 10.65 percent, germacrene 9.73 percent, caryophyllene 5.84 percent, and dodecane 4.54 percent) and 28.3 percent oxygenated compounds (octadecanoyl 16.7 percent, and dodecane 4.54 percent) and 28.3 percent oxygenated compounds. Prenylated flavonoids (5, 7, 4′trihydroxy 8 phenyl flavanone, 5, 4′ dihydroxy 7 methoxy 8 phenyl flavanone, 5, 7, 4′ trihydroxy 3′, 8 diprenylflavanone and 5, 7, 4′ trihydroxy 3′, 5′ diprenylflavanone) were found in methanolic extract of neem flowers and exhibited antimutagenic activity for TrpPI [47]. Other compounds include flavanones, triterpenoid (trichilenone acetate), nimbaflavone, 3′prenylnaringenin, and 4(2hydroxyethyl) phenol constituents contained in neem flowers. Using various chromatographic techniques, oil isolated from neem seeds and kernels by use of methanol were found to contain tetranortriterpenoid alcohol. Copaene, Cubebene, humulene, cardionene, and a variety of sesquiterpenes were also included in oil of neem tree's flowers, all of which had antimicrobial properties. As determined by capillary GC/MS, the neem tree's seeds contained highly volatile organo-sulphur compounds, especially dinpropyl and n-propyl-1, propenyl groups containing di, tri, and tetra sulphides. Salannin extracted from neem seed oil by HPLC "high-performance liquid chromatography". Elemene were the most common compounds found in the essential oils of neem. In Figure 2.2 the significant antibacterial volatiles derived from neem [47].

Table 2.2: Phyto-compounds isolated from *A. indica* [45, 47]

PARTS USED	PHYTOCHEMICALS ISOLATED	NATURE OF EXTRACT
ROOTS	Nimbilin and nimolinin	Dichloromethine and methanolic extracts

PARTS	PHYTOCHEMICALS	EXTRACT
LEAVES	Stigmasterol, terpinen-4-ol, sugiol, 4-cymene, nimbiol, a-terpinene, and vitamin E Steroid, glycoside, flavonoids, triterpenoid carbohydrate, alkaloids, and antiquinone Zafaral, meliacinanhydride, nimocinol, and isomeldenin Nimonol Nimbothalin and n-tridecyl benzene Isoprenoid, flavanone 08.3'-di-isoprenyl-5, 7-dihydroxy-4-methoxyflavanone), nonisoprenoids, and meliacin 22.23- Dihydronimocinol and desfurano-6-a- hydroxyazadiradi one Mellatetraolenone and odoratone Volatile compounds Tetracyclic triterpenes a-Linolenic and Nimonol	Dichloromethane and methanolic extracts Chloroform, aqueous ethanolic extracts Ethanolic extract Ethanolic extract Ethanolic extract Ethanol extract fractionated fractioned with chlorofors and n-butanol Methanolic extract Methanolic extract O extracted with steam Ethanolic extract nehexane extract Methanolic extract
FLOWERS	Prenylated flavonoids Tkivetine were methylandwonde dieck azadizipl Sesquterpenes aromatic compound, fatty acids and fattydesten, stepids, and hewdrocabons	Methanolic extract Methanolic extract n-hexane extract Methanolic extract
FRUITS	Aubarone azadirone, and soazidi onol Mahmoodin anadiciacht and inahessdin Samurrain, aradrols; and, acadradnol and azadiranal Limocinone mocin A impon Bimocnol, and lirocini	Ethanolic extract Ethanolic extract Ethanolic extract

PARTS	PHYTOCHEMICALS	EXTRACT
SEEDS	Genistein 7-0-glucoside and (-)-epi-catechin Tetranortriterpene alcohol Organosulphur compounch Salannin Azadirachtin M. azadirachtin N. 11-epi-azadiracht in H triterpenoid1a7a- diacetoxyapotirucal-14-ene-30.21. 2224.25-pentiol), odoratone, and 28.38 48. trihydroxypregnan-16-one 11-Hydroxyazadirachtin-B, 1-tigoyl-3-acetylazadirachtinin 1.2-diacetyl-7-tigloy-12-hydroxyvilisinin, 23-desmethyllimacin-B 1a-Methoxy-1. 2-dihydroepoxyazadiradione, 1828-diepoxyazardiradione. 7-acetylneotrichienone, de sacetyl-7-benzoylaradiradione 7-desacetyl-7 benzoylepoxyazadiradione, 7-desacetyl-7 benzoyi-gedurin Azadirachtin Deacetylarad rachtinol 1020-Epoxy-178-hydroxyazad radione 10, 20-epoxynimolicinal and 7-deacetylnimolicino along with epoxyazadiradion 170-hydroxyadiradione, gedurin, nimbin, remolicinal	Methanol, n-hexane, and ethyl acetate extracts Seed oil Diethyl ether extract Steam volatile extract n-hexane Methanolic extract Methanolic extract Petroleum ether extract dissolved in methanol Dichloromethane extract Methanolic extract Seed oil Methanol extract

Several bioactive compounds were found in the extract of seeds and kernels of neem, including azadirachtin M, azadirachtin N, 11-epiazadirachtin H, and triterpenoid(1, 7diacetoxyapotirucall-14-ene3, 21, 22, 24,25pentaol) as well as known

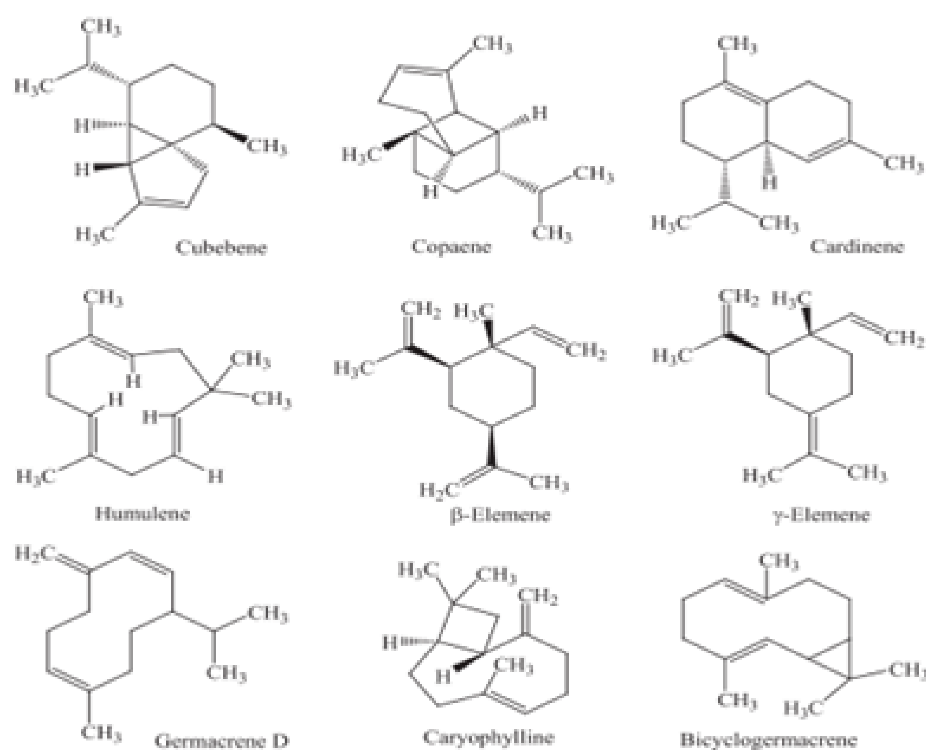

FIGURE 2.2: Structure of anti-bacterial volatile from *A. Indica* [48]

compounds such as odoratone and 2, 3, 4 trihydroxypregnan 16 one. In a previous phytochemical analysis of seeds, 11 hydroxyazadirachtin-B, 1 tigloyl 3 acetylazadirachtinin 1, 2 diacetyl 7 tigloyl12hydroxyvilasinin and 23-desmethyllimocinB were found. According to [49] 1 methoxy1, 2 dihydroepoxyazadiradione, 1, 2 diepoxyazardiradione, 7 acetylneotrichilenone, desacetyl7benzoylazadiradione, 7 desacetyl 7 bbenzoylazadiradione, 7 desacetyl 7 bbenzoylazadiradione, 7 desacet, and 7-desacetyl-7-benzoyl-gedunin were isolated from the neem seeds [47]. The oil of *A. indica* was used to extract tetracyclic triterpenes including meliantriol and nimolicinol. Tetracyclic triterpenes including nimbocinone, nimocin, azadirachtol, and nimocinol were present in the fruits and leaves of neem. The occurrence of linolenic acid in the tree's fresh leaves had been reported. The spectral studies such as COSY and NOESY NMR spectroscopies were used to determine the structure of nimonol derived from neem leaves. Another study provided that the percentage of azadirachitin and salannin in fruit lowered upon ripening, affecting the bitterness of fruit. The root bark was discovered to contain nimbilin and nimolinin, two novel terpenoids [50].

Azadirachtin had been extracted from the neem tree seeds in several studies. Nimbidinin and nimbidic acid had been extracted from neem seed kernel extracts. The extract obtained from the seeds of neem contained tetranortriterpenoid (11-epi-azadirachtin H), salannol-3-acetate, 11-epi-azadirachtin D and nimbanal [51] explored in dried kernels of neem tree about the presence of the 3 new limonoids like 1-benzoyl-3-deacetyl-1-detigloyl, salannin, 7-tigloyl-12-oxovilasini, azadiralactone and triterpenoid, azadirahemiacetal. The seeds extract contained tetranortriterpenoid (11 epiazadirachtin H), 11 epiazadirachtin D, nimbanal, and salannol3acetate [52] investigated the existence of three new limonoids in the dried kernels of the tree, including 1benzoyl3deacetyl1detigloyl, salannin, 7tigloyl12 oxovilasini. Deacetylazadirachtinol, a novel limonoid found in seed oil extract, is a strong inhibitor of insect ecdysis. Elemene (20.8 percent), germacreneB (20.3 percent), trans caryophyllene (13.5 percent), hexadecanal (12.8 percent), and methyl linoleat were discovered in leaf oil recently 10.5 percent [53].vThe compounds: epoxyazadiradione, 17 hydroxyazadiradione, gedunin, nimbin, and nimolicinol, oils from neem seeds consisted of 1, 2 epoxy 17 hydroxyazadiradione, 1, 2 epoxynimolicinol, and 7 deacetylnimolicinol. The neem stem bark consisted of nimbinone, isonimbinolide, nimbionone, nimbionol, and nimbonolone, whereas the root bark contained nimblin and nimolinin. In other studies, margocin, margocinin, margocilin, and nimbidiol were found in the bark of root. The neem tree's heartwood consisted of a useful bioactive 24methylenelophenol [22].

2.3 Active Components

Neem was the source of various ingredients that play a therapeutic role in health management. Azadirachtin is the most active constituent derived from neem and others are sodium nimbinate, Nimbin, gedunin, nimbolinin, nimbidin, quercetin, and salanin. The chief constituents of leaves are nimbanene, nimbandiol, ascorbic acid, nimbin, 6-desacetylnimbinene, nimbandiol, n-hexacosanol and amino acid, 7-desacetyl-7-benzoylgedunin, 7-desacetyl-7-azardirone and nimbol [54, 55][8, 9, 15]. According to [8], b-sitosterol and Quercetin, polyphenolic flavonoids was isolated from neem leaves and show antibacterial and antifungal characteristics [56] . The seeds contain useful copounds like azadirachtin and gedunin [57].

Neem has significant importance in disease prevention and control. The exact mechanism involved in pathogenesis prevention is not clear till now. Neem plays a therapeutic role due to antioxidant activity performed by important compounds such as nimbolinin , nimbidin, salanin, azardirachtin, nimbolinin and quercetin. Neem parts show an antimicrobial role by breaking the cell wall of Azadirachtin, in seed, was a complex tetranortriterpenoids limonoid that has antifeedant and toxic effects among insects [58] It was provided from literature that extract of leaves exhibited anti-bacterial properties with the greatest zone of inhibition noted 100% concentration against MRSA and Staphylococcus aureus [59].

The free radical scavenging property of neem play a significant role because of high amount of antioxidants. Nimbolide and Azadirachtin exhibited antiradical sedative potential and scavenging properties which is in this order nimbolide $>$ azadirachtin $>$ ascorbate [31].

1. In the management of cancer, neem ingredients show effective role through cell cycle regulation. It modulates the transcription factors (NF-KB), tumor suppressor gene(P53, pTEN),Apoptosis(bax, bid) and Angiogenesis (VEGF).
2. The anti-inflammatory role of neem is due to the regulation of pro inflammatory enzyme activities like lipoxygenase and cyclooxygenase(COX) enzymes.

2.3.1 Pharmacological Attributes

The active constituents play role in disease cure through antioxidative enzymes activation, bacterial cell wall breakdown, and chemo preventive role through regulation of cellular pathways [6].

2.3.1.1 Antibacterial Activity

Certain bioactive compounds of neem like triterpenoids, steroids, reducing sugar, sesquiterpene lactones, carbohydrates, tannins, flavonoids, triterpenoids were involved in anti-bacterial property [60], [57, 59] [61–63]. The growth of *S. pyogens*, *E. coli*, *M. coccusluteus*, *Enterobacter*, *B. subtilis*, *P. aeruginosa*, *K. pneumonia* are inhibited by acetonic, methanoic, and aqua extract of neem leaves [18].

Another study from the literature revealed the neem antibacterial effects against several bacteria, including *P.aeruginosa*, *Klebsiellaozaenae*, *S. aureus*, *S. typhi*, *P. mirabilis*, *S. paratyphi B*, and *E. coli*. For *P. aeruginosa*, *K. ozaenae*, and *S. aureus*, *E. coli*. The minimum bacterial concentration (MBC) was 50 mg and minimum inhibitory concentration (MIC) was 5 mg. The antimicrobial property of neem extract dissolved in dimethyl sulphoxide against S.mutans, S. aureus, and E.faecalis were provided [64] with sodium hypochlorite/chlorhexidine medication did not vary statistically, implying that it could be a suitable alternative to other root canal irrigates [65, 66]. The neem crude bark and seed extract stopped the growth of both types of bacteria i.e., gram-negative and gram-positive bacteria [67]. Against adult mouth bacteria the extract of neem leaves, bark, seeds, and fruit was very effective by using the agar well diffusion process. Several pathogens, including viruses, bacteria, and fungi were shown to be inhibited by oil extracted from seeds of neem [68, 69]. Antibacterial activity was demonstrated against V.vulnifcus by a mixture of tree oil nanoemulsion and tween 20/water. The seed oil ethanolic extract (9%) had a good antibacterial capability against *E. coli*, with a zone of inhibition (24 mm) [70].

Furthermore, flower extracts were found to be bactericidal against *B. cereus*, *S. aureus*, *L. monocytogenes*, *E. coli*, and *S. infantis*. Many studies [71, 72] verified the bactericidal effects of *A. indica* extracts. The antimicrobial and antioxidative properties of neem were attributed to several secondary metabolites (flavonoids, nimbolide, azadirachtin, and so on). Nimbolide, active antibacterial compound extracted from leaves, showed a wide ranged antibiotic for *E. cloacae*, *B. subtilis*, *E. faecalis*, *S. epidermis*, *E. aerogene*, *S. typhimurium* [73]

The *Pseudomonas spp.*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Klebsiella sp.*, *E. coli*, *Salmonella sp.*, and *S. aureus* were all immune to neem tree's synergistic influence in combination with other plants such as *Psidium guajava*, *Camellia sinensis*, and *Calendula officinalis* [74] with exception of *V. parahaemolyticus*, *P. aeroginosa*, and *Aeromonas hydrophila*, *P. guajava* and *A. indica* extracts. The antimicrobial activity of gram-positive than gram-negative bacteria was more significant. The antibacterial ability of neem extracts was found to be unaffected

by temperature and pH. The ethanolic extract of neem fresh leaves had high antibacterial property against *E. coli*, *S. aureus*, and *P. aeruginosa*, with inhibition zones of 21 mm, 16.5 mm, and 10 mm, respectively, then dry leaf extracts [75, 76]. In the case of methicillin-resistant S. aureus, ethanolic extract of neem leaves had higher MIC values than twigs, while MIC values were similar for *E. coli*. For *P. mirabilis* and *E. faecalis*, the MIC values were 6.25 and 12.5 mg/ml [4, 74].

Aqueous extract of neem leaves was more effective in controlling *E. faecalis* growth than sodium hypochlorite (NaOCl) and antibiotics [77, 78]. The antimicrobial potential of combined leaves extract and mucoadhesive dental gel formulation (25 mg/g) against *S. mutans* and *Lactobacilli* species was significant similar findings obtained by using neem tree's bark and twig [79–81]. The neem non-absorbable oil chip (10%) demonstrated antibacterial activity against a periodontal pathogen, *P .gingivalis* [82, 83].

Similarly, results obtained for extracts of leaves of *Azadirachta indica*, *C. sinensis*, and *M.carundinacea* [84]. The antimicrobial properties of hand wash containing extracts from O. sanctum, Aloe vera gel, *S.mukorossi* fruit, *A. indica*, Eucalyptus species, and citrus fruit were tested against pathogens like *S. aureus*, *K. pneumonia*, and *S. typhimurium*. The antimicrobial activity of *A. indica* leaves extract was found to be important among all herbal formulations [12, 66].

Silver nano-particles from neem leaf extract inhibited causing strains of bacteria like *P. aeruginosa*, *S. typhi*, *S. aureus*, and *S. pyogenes* [59, 85]. Several studies use leave extracts demonstrated possible antibacterial activity against *B. pumilus*, *Pseudomonas testosteroni*, *S.epidermidis*, *K. pneumoniae*, *B. subtilis*, *Proteusmorganii*, *M. flavus* and *E. coli* [84] [53, 86–90]. Several studies had also shown that various parts of this magical tree had antimicrobial properties. So, based on literature neem could be used further against gram-positive and negative bacteria due to its antibacterial properties [64, 91, 92].

2.3.1.2 Antifungal Activity

The neem oil and leaves extract showed fungicidal property in various studies. The polar and non-polar extract of the stem, fruit, and leaves by methanolic

and chloroform extract showed fungicidal activity against R.solani [47]. The seed extract used in growth medium had a fungistatic effect against S.fuliginea. It has also been shown that 24-day-old neem leaves and twigs were more successful than older leaves and twigs. In mango trees, the synergistic effect of A.indica, Datura stramonium, and Calotropis polar extracts was effective against F.mangiferae and increased floral yield [44]. The neem seed oil, aqua, and ethanolic extract of leaves presented a major reduction in P. oryzae growth. The antifungal activity of seed oil was found to be the highest, followed by ethanol, cold water, and hot water leaves extracts. The seed-borne fungi Rhizopus, and Aspergillus was strongly inhibited by aqua and alcoholic extract of neem.

The neem seed oil, aqua, and ethanolic extract of leaves presented a major reduction in P. oryzae growth. The antifungal activity of seed oil was found to be the highest, followed by ethanol, cold water, and hot water leaves extracts. The seed-borne fungi Rhizopus, and Aspergillus was strongly inhibited by aqua and alcoholic extract of neem. The neem seed kernel of ethanolic extract *M. fructicola*, *P. expansum*, *T. roseum*, and *A. alternata* were found infecting plums [47]. The neem tree's seed oil hindered the growth of *Aspergillus* and *Aspergillus* alternata spores. Seed oil from neem tree inhibited the growth of *Aspergillus niger*, *Aspergillus flavus*, *Fusarium oxysporum*, *Fusarium moniliforme*, *Fusarium nivale*, *Fusarium semitectum*, *Drosophila hawiiensis*, and *Aspergillus alternata* more effectively than oil extracts from *Brassica campestris*, *Nigella sativa*, and *Ferula ass.* The essential oils of *C. citratus*, *E. camaldulensis* and *A. indica* are combined to control seed-borne infections due to *C. graminicola*, *P. sorghina* and *F. moniliforme*. The fungicidal activity of ethyl acetate extracts of leaves and fruits against *A. solani* was strongest, followed by methanolic extract [93]. Brown spot disease of rice, caused by C.miyabeanus, was inhibited using ethanolic extracts of leaves and seed oil. Furthermore, oil extracts were found to be more effective in reducing disease symptoms [88].

The high-performance liquid chromatography and NMR discovered the anti-fungal activity of nimonol [49]. In vitro application of A. indica and Nicotiana tabacum extracts revealed that *N. tabacum* had a fungi toxic effect on *A. viridae* and *P.*

digitatum mycelia development. The *A. indica* extract showed a lowering of growth in a manner of concentration-dependency [94].

2.3.1.3 Pesticidal Activities

The neem compound Azadirachtin that impaired cell division and protein synthesis by indirect (0.3–0.99 mg/L) and direct (0.35 and 1.28 mg/L) effects on Culex pipe were studied, that provided muscle flaccidity, necrosis of midgut cells, the loss of nidi (regenerative cells), and a lack of midgut enzyme activity. The neem compounds also provided a decrease in adult mosquito fecundity and significantly increased Culex sterility and larval developmental stage. Pesticides based on neem demonstrated efficacy by reducing detoxifying enzymes or breaking resistant compounds by blocking enzyme production or reduce the midgut cell turnover rate, depending on the mechanism of resistance [95, 96].

Natural pesticides were environmentally friendly, biodegradable, and were effective against pests. One form of biopesticide based on the neem tree, had a variety of insecticidal, antifeedant, toxicologically repellent, sterility inducing, and insect growth-inhibiting properties. Nano-gum (10 ppm) made from the gum of *A. indica* had been evaluated as a new generation biopesticide against *H. armigera* (Hub.) and *Spodopteralitura* [84, 97]. Azadirachtin had a direct lethal effect on the pests' tissues through the endocrine system [85].

2.3.1.4 Anthelmintic and Anti-leishmaniases Activity

Leishmaniases were parasitic diseases caused by the parasite Leishmania. Anti-leishmaniases behavior was observed for several *A. india* extracts (leaves, seeds, bulbs, and fruits). In vitro study of dichloromethane and aqueous extracts of leaves and seeds prepared with ethanol against promastigotes and *L. amazonensis* with minimal cytotoxic effects, the ethanolic extract was found to be effective against leishmaniases [97, 98]. Aqua and crude extracts of neem at higher concentration show high anthelmintic activity to infected sheep with GIT nematodes (1–3 g/kg of body weight) [68, 99]. Several studies used animal models and revealed successful results for treating infections and parasitic-based skin disease [90, 100].

2.3.1.5 Antidiabetic Activity

Hyperlipemia, glycosuria, ketonemia, negative nitrogen balance were all signs of diabetes mellitus (DM). Diabetes Mellitus improves peripheral insulin resistance by lack of control in blood glucose concentration and is related to blindness, kidney failure, cardiovascular disease, and nervous system disorders. It provided a lifelong burden to the patient due to disease progression therefore cost-effective treatment was very essential. Among various pharmacotherapies and methods that had been developed the neem extracts were very interesting [22, 25]. There were two types of diabetics. The neem extracts provide controversial results in both types of diabetes but due to toxicity effects, the direct use of neem extracts is avoided. The pancreatic B cells were unable to produce insulin in early-onset and were referred to as Type I diabetes. However, a genetically susceptive individual due to excessive calorie intake and sedentary lifestyle were unable to intake glucose in muscle and fat cells that leads to the condition of insulin resistance that was referred to as diabetes type II. It was due to the downregulation of NADPH and glucose-6-phosphate dehydrogenase reduction that leads to improper production of ROS and intracellular deduction of NADPH by the time [69, 101, 102]. The process leads to induce pro-inflammatory signaling molecule induction like IL-6 and TNF-alpha through oxidative state disruption [79, 103]. The overall mechanism leads to a diabetic state by insulin resistance pathways [69, 89, 104]. People prefer herbal treatments for diabetes (80%) all over the world due to lesser side effects [48, 99]. In various forms, *A. indica* is used for hypoglycemic and antihyperglycemic purposes [48, 99]. The increased production of insulin [105] inhibition on glucose metabolism by epinephrine action on enhanced utilization of peripheral glucose. It has been discovered that active phytochemicals, such as Azadirachtin, can help postpone or avoid disease onset [97]. Specifically [89, 106] provided that the anti-oxidative system was recovered and the kidney along with liver show retardation. They provided that bark and leaf extracts had similar glucose homeostasis as shown by control or standard use of insulin. Similarly, the effect of neem seed enriched extract epoxy-azadiradione caused a 37% reduction in glucose level during some hours. The long-term study provided that after 15d period neem extract modulate

sugar level in blood by 800 mg/kg sugar in blood. The tested model had a 50% reduction with the maintenance of 300 mg/dl glucose level. Other studies also provided similar results after using chloroform-based extract. The experiment by using islet cell morphology, insulin level, and glucose testing level of insulin was similar to the control group after treatment. The cells entered in the state or perhaps apoptosis and altered morphology. So, neem compounds had confident results for pancreatic health and glucose reduction, along with kidney damage and liver retardation in antioxidant system [12, 106].

2.3.1.6 Antiulcer, Nephroprotective, Hepatoprotective Activities

The unregulated production of acid by parietal cells of the gastric mucosa causes hypersecretion throughout the stomach. Peptic juice production, oxidative damage from reactive oxygen species, and apoptotic cell death were all-important during ulceration. The ulceration can be regulated for effective healing through inhibiting hypersecretion of gastric acid, scavenging reactive oxygen species, and blocking apoptosis. Aqueous extract of leaves given to three groups of Wistar rats at doses of 150, 300, and 600 mg/kg body weight reduced ulceration caused by pyloric ligation, aspirin, and cold restraint stress in a dose-dependent manner [64].

As a result of substantial reduction in total phenolics and vitamin C percentage, the antioxidant capacity of stored aqueous crude extract from *A. indica* leaves lowered by time and temperature. Nimbidin, a compound extracted from neem demonstrated antisecretory activity in rats and cats with a ligated pylorus. Azadiradione, a cytoprotective and antisecretory neem compound isolated from ethanolic extracts of seeds, exhibited effective antiulcer properties by inhibiting H+/K+ATPase through its cytoprotective and antisecretory effect in cold restraint, aspirin, alcohol, and other conditions. In a cisplatin-induced model, extracts of leaves showed strong defense from ulcers by lowering serum alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transpeptidase, alkaline phosphatase, complete bilirubin, creatinine, uric acid, and urea. The results led to the inference that leaves would defend against hepatotoxic and nephrotoxic effects [107].

2.3.1.7 Antioxidant Property

Reactive oxygen species (ROS) or free radical acted upon many biological molecules and causes inflammation through unleashing the cell in a state of oxidative stress by taking electron out from entering the stable state [106]. So there arose a requirement for compounds that block or prevent oxidative stress by neutralizing or stabilizing these free radicals that cause multiple diseases. The action of natural antioxidant defense was supplemented by these antioxidant molecules: catalase (CAT), glutathione peroxidase (GPX), superoxide dismutase (SOD), nitric oxide dioxygenase (NOD). Usually, the simple way of providing these compounds to the body was through a supplementary diet. One process to introduce supplement antioxidants was through derivation of neem natural extract by oils, teas that were the cost-effective and simple way the safety and efficacy of extracts were artisanal as the potential benefits vary from preparations to preparations, but it was still considered safe due to tradition medical importance. However certain natural compounds alter the pathological states. With time the antioxidant activity of neem was provided to check the impact on body natural defense. One study provided a potential extract from neem compounds from methanol and leaves. In that study, they conducted their work on rats for seven days as pre-treatment at 100-200 mg/kg comparison to untreated vitamin C(antioxidant) treated animals, in the model of induced intestinal ischemic-reperfusion injury (IIRI). The extract group reduced several inflammatory markers such as myeloperoxidase and IIRI rats had decreased expression of the extracellular regulated kinase (ERK1/2) in serum.

For IIRI the level of nitric oxide was (.025 μmole/l) while for non-IIRI the NO continued at a steady level (vitamin C .042 μmole/l, control .036 μmole/l and extract .034 μmole/l). Furthermore, the level of GSH in the extract group was increased that causes recovery of glucose-6-phosphate dehydrogenase (G6PD) hence strengthen the natural defense mechanism [11, 69]. Other studies induce a model of colitis in rats by using acetic acid by comparing no extract to extract up to 14 days, which provided information about the reduction in inflammation and colonic mucosal tissue damage at a microscopic and macroscopic level by measuring GSH,

SOD, and CAT the level of enzyme reduction in the colitis model was 85%, 61%, and 46%, respectively, however, the level of CAT and SOD was the same as that of the control group despite GSH 85% recovery level. The rats without extract treatment gain weight without disruption of food supply comparative to extract treated and control group by providing evidence of natural antioxidant system steams by extract consumption. From another study, the concentration of phenolic contents was 20% higher in neem-rich yogurt as compared to conventional yogurt. After laboratory testing of neem enriched yogurt, the DPPH inhibition capacity was 53.11 μgGAE/ml (day 28) vs 35.9 μg GAE/ml comparison to plain yogurt. The maximum inhibition provided by these molecules through testing was alpha-glucoside (15.2%), angiotensin-converting enzyme (48.4%), alpha-amylase (47%) in hypertension and diabetes. So concluded that the neem enriched yogurt increases natural scavenging activities in the body [108].

2.3.1.8 Cardioprotective and Neuroprotective Properties

The use of an aqueous extract of neem leaves before doxorubicin treatment protected tumor-bearing mice from cardiotoxicity. Antioxidants in the extract had a modulatory effect on the body's defensive system, preventing cardiotoxicity [92]. The cardioprotective ability of aqueous extract of leaves against isoprenaline-induced myocardial infarction in rats was investigated by calculating total cholesterol and triglyceride levels that were higher in the isoprenaline control group, while HDL levels were lower. However, combining neem extract with vitamin E at doses of 250, 500, and 1,000 mg/kg strongly improved all hemodynamic, biochemical, and behavioral outcomes. The cardiovascular system of anesthetized guinea pigs and rabbits was covered by neem crude leaf powder, which caused hypotension and reduced the negative chronotropic effect in a dose-dependent manner. Different extracts assisted in the management of cardiovascular conditions by raising the lipid profile. The tree was high in flavonoids, which inhibit LPO and reduce the symptoms of neurotoxic [59]. In rat 12 cell lines the neem seed flavonoid was tested for the neuroprotective property. The neem ethanolic extract of seeds had neuroprotective effects when added to neurons that had been pre-exposed to the neurotoxin (6-Hydroxydopamine). The result provided that, neem tree extracts

can be used to treat neurological conditions [43]. When rats were given 500 mg/kg of a finely ground whole tree for 15 days, it greatly reduced the functional disruption caused by cerebral hypoperfusion. Treatment with A.indica successfully reduced reactive changes in brain histology. Along with inhibition of interleukin-6 and tumor necrosis factor (TNF), the extract of leaves caused significant reductions in cell inflammation, reactive oxygen and nitrogen species concentrations, and tumor necrosis (TNF) and interleukin-6 factors [48, 105]. Seed extracts containing flavonoids had neuroprotective effects on the rat PC12 (Pheochromocytoma) cell line. The neem seed extracts act like supplements for Parkinson's disease and many other neurological disorders [43].

2.3.1.9 Antifertility Activity

In the female reproductive tract, numerous contraceptives with diverse modes of action are produced from the natural and synthetic sources of neem due to reversible infertility and good spermicidal activity [109]. At a dosage of 10 mg for 15 days, azadirachtin showed no oviposition and signs of ecdysteroids in the ovaries of Locusta migratoria females. Interference of azadirachtin with the neuroendocrine regulation of hormone synthesis mediated oogenesis inhibition and the presence of ecdysteroid [110]. The investigation of neem seed oil extract was done quantitatively in female albino rats on follicular growth by using polar and non-polar solvents. At various stages of growth and differentiation, the number of follicles was found to be significantly reduced. The result provided that the effects of azadirachtin and A.indica oil on *C. formosanus* oviposition, feeding activity, and tunneling. *C. formosanus* dodged contact with *A. indica* oil-treated sand/paper and loss of appetite. In B.microplus, the effectiveness of herbal acaricides derived from *A. squamosa* and *A. indica* was compared, the most effective infertility agent was *A. indica*, which did not affect the ovaries or change the oviposition of females [107].

2.3.1.10 Anti-inflammatory Activity

Non-steroid anti-inflammatory drugs (NSIADs) contain side effects such as speeding up osteoarthrosis despite minimizing inflammation. The extracts and decocting Phyto-medicines were used as anti-pyritic, interoceptive, and analgesic agents

to minimalize side effects [111, 112]. In rats ethyl acetate,methanoic and n-hexane leaves extract of neem provided anesthetic and anti-inflammatory activity relative to indomethacin [113]. Similarly, anesthetic and the anti-inflammatory role was also provided by aquas extract of neem leaves after chemical and thermal mediated models in rats [95].

The neem extract act as anti-inflammatory agents [114, 115]. Inflammation is a condition that is associated with several diseases like diabetes and cancer as well as in other states such as food digestion and drinking alcohol [116]. Limonoids were the major bioactive compound found in neem. The limonoids are known as furanolactone as anesthetized due to their inhibitory property and reduction in inflammatory mediators by endogenous opioid pathways [109, 115]. Soars of liminoid extract of neem at concluding effective dosage 120 mg/kg when tested on damaged rat's paws fibrovascular tissue and edema growth were inhibited by through particular inhibitory effect in tumor necrosis factor-alpha (TNF-alpha), interleukins, and inflammatory molecules. Several other studies also investigated and corroborated the mechanism of limonoids anti-inflammatory activity. The detail mechanism about anti-inflammatory role of neem limonoids was provided by several studies [117–119]. Another compound such as epoxy-azardirone had an anti-inflammatory effect. This compound showed pathogenesis through cytotoxic potential by modulating macrophage migration inhibitory factors; ability to translocation of NFKB, inhibition of tautomeric activity, and prevention of pro-inflammatory cytokines such as IL-B, IL1-A, IL1-6, and TNF-Alpha [106, 107]. In the body, inflammation activated the cyclooxygenase pathway and activated inhibition of cyclooxygenase 1 and 2 (COX1, COX2) by neem [81].

From this information of neem Phyto-compounds, the modulation of inflammation was associated with eicosanoid metabolism (thromboxane and prostaglandin) that was an important step for converting arachidonic acid to PGE2 and PGH2 [118]. The platelet-activated factors in macrophages and monocyte were expressed by COX2 an enzyme that was stimulated by platelet and IL-1 [80, 120, 121]. From the above-mentioned studies the epoxy-azadirone and level of transcriptional factor that mediated inflammatory cytokines like IL-6, IL-1 and TNF-a provided

evidence about anti-inflammatory activity [120]. The anti-pyretic effect was produced by the neem extract that interfere IL-1-COX stimulation by the production of antipyretic effect [80]. It also inhibits NF-KB nuclear translocation by reducing the inflammatory response and hence reduces TNF-a and cytokines by serving as a mediator in the cancer signaling pathway [80]. So neem extracts were responsible for proinflammatory reactions in various diseases by inhibition of proinflammatory cytokines that have an inhibitory effect on macrophage migration diseases [122].

2.3.1.11 Anti-cancer Activity

To determine the anti-cancer activity of Phyto-compounds and medicinal plants several studies had been conducted in the last years [13, 85, 101, 123, 124]. It interferes with the growth or apoptosis by multiple pathways even had a chemo-protection effect [73]. One of the studies was conducted to determine the chemo-protective effect in neem compounds like limonoid, azadirachtin and nimbolide by using the hamster buccal carcinogenic model. It provided that these compounds played a significant role in the suppression of NF-kB pathways. By expression profile of Fas, Bid, PCNA, cytochrome c, Bcl-2, P53, cyclin D1, surviving and caspases the overall reduction was reported [86]. Some other studies showed the anti-cancer effect of compounds derived from neem limonoid. The study provided that these limonoid 15-o-deacetylnimbolindin B and 1-15-o-deacetylnimbolindin B hinder cell growth by suppression of wnt-B, NFKB, and JACK/STAT pathways in human adenocarcinoma cervical [85, 87]. There were two more cytotoxic compounds azardirone and nimbolide provided by [74] that cause reversion-inducing Kazal motif due to cysteine-rich protein and induced ROS mediated apoptosis by Akt/PI3K signaling. In neem leaf ethanolic extracts the alkaloid-derived limonoid and azadiramid-A induced apoptosis and stop cell growth in estrogen-dependent MCF-7 and estrogen-independent MDA-MB-231 cell lines [87, 117]. Furthermore, by using azadiramid-A, the level of pro-apoptotic signaling molecules BCL-2 associated with BAX and Bad, Cyto C were elevated by triggering of caspase-3 activity and FasL, Bcl-2, Bcl-xl, FADDR, and TRAIL were downregulated [57, 104, 124]. The rate of cellular proliferation rate also decreased by using neem leaf ethanolic extract through IGF signaling molecules by apoptosis-inducing activity [57, 104].

Some other compounds like NLGP modulate Effector T, NKT and NK activation by inhibiting T cell regulation, maturation of dendritic cells, and macrophage maturation. These compounds further normalize tumor immune microenvironment by preventing effector T cell depletion through regulation of cytokines-chemokines (VEGFR2 and reducing CD31) [88].

2.4 Safety, Toxicities, and LD50 Values of Neem

The measurement of toxicities was very important in health management before use natural compounds. Based on various animal and clinical models the neem and its ingredients show safety and some have few adverse effects. The poisoning of neem oil causes hepatic toxicity, encephalopathy, vomiting, and metabolic acidosis. At a low dose, the administration of neem leaf scarp causes an anti-inflammatory effect and not side effects by a high dose. A study conducted on the rat's model shows that does not show side effects even at the dose of 5g/kg. The toxic analysis was performed on rabbits developing body weight in control and test and during the administration of neem leaf extract [90]. The LD50 value of neem oil is 31.95g/kg [13].

The aqueous extract of neem leaves on dose-dependent manners revealed LD50 4800 mg/kg toxicity in chicken. The lethal dose LD50 for stem bark and neem leaf were 489.9 mg/kg and mg/kg 31.62 [13]. The LD value was analyzed and LD50 and LD90 value was 169.8 ug/fly and 8.4 ugs/fly of neem extract. The LD50 test for acute oral toxicity is 13g/kg weight [100].

2.5 Strategies for Targeting Cancer Metastasis

The major cause of cancer-related death was due to metastasis. The lack of knowledge and a clinical trial that targeting metastasis were the major obstacles that govern the process of metastasis. The phenotypic and genetic differences between metastatic/circulating and parental cells should consider during targeted therapies for cancer metastasis [49, 93, 97, 125]. The terminal labels were associated with diagnosis of metastatic cancer. Furthermore, preclinical prevention had been demonstrated but due to poor therapeutic strategies and clinical trials hindered

this process [48, 99]. In metastatic melanoma, the advancement in immunotherapy had improved patient and survival outcomes. The survival of metastatic prostate cancer was inhibited by the enhancement of novel androgen receptor inhibitors. However, the consistency in survival benefits of patients with metastatic breast cancer had been failed due to long term follow-ups [93]. In metastatic cascade, the targeted strategies for these pathways had been explored and provided. The cancer cells seeding could be targeted by inhibiting intercellular crosstalk by ECM adhesion molecule, EMT, intratumorally interaction, the release of proteases, and intravasation. During diagnosis, the cancerous cells are already seeded and colonized at the circulatory system or distant site. So, the most plausible therapeutic strategy was targeting metastatic colonization due to correlation with the clinical scene. The potential target of metastatic colonization was dormancy. Some therapies helped in sustaining that dormant state while the rest of the therapies were designed for targeting the G0 stage of tumor cells. However, on the other hand, monoclonal antibodies targeted single cancer cells at the dormant stage [105]. The lack of patient accrual the trial of targeting patient tumor-secreted factors along with exosome affinity plasmapheresis for trapping tumorigenic or immunosuppressive material was terminated. So novel agents that cross BBB must be designed and tested in the brain to track their crossing [93]. In this regard combine therapy by using *A. indica* compounds that target multiple signaling pathways simultaneously for effective inhibition of metastasis in cancer cells would be effective [126].

2.6 Targeted Pathways

Phytochemicals and their derivative provided an improved option for efficacy in cancer treatment due to fewer side effects among patients. *A. Indica* (neem) is the storehouse of various phytochemicals [20, 21]. The active compounds of *A. indica* manifest cancer signaling pathways through targeting gene expression. The major pathways targeted by *A. indica* compounds were apoptosis and inflammatory pathways that were involved in anticancer activity [13].Apoptosis is crucial for homeostasis and development by controlling cell proliferation and growth [127]. To be certain, Apoptosis and necrosis may be used interchangeably but occurs at the same time, but they are triggered was dependent on the intensity and level

of stimuli, trauma, infection, toxic reduced amount of ATP when caspases fail to work. So, based on this apoptosis was a defined process that leads to activation of cysteine protease to perform the process that allows a cell to demise to equate the level of cell proliferation for development and homeostasis. So, many pathways trigger apoptosis either intrinsic through pro-apoptotic proteins or extrinsic through the involvement of ligand [111]. Apoptosis was triggered by DNA damage because of several diseases like spinal muscular atrophy, toxin, stress, and Alzheimer's disease, uncontrolled cell proliferation by demonstrating importance for defending against diseases and maintaining cell population [112]. It had been also provided that several conditions initiate phenomena like hormonal release and administration of therapeutic drugs. On another hand, Inflammation was associated with the immune system's response to harmful stimuli such as damaged cells, pathogens, irradiation, or toxic compounds [113] by initiating the healing process and removing injurious stimuli [30, 128]. Usually, during acute inflammatory responses, molecular and cellular events and interactions provide efficient threats of minimizing infection and injury. The uncontrolled acute inflammation may become chronic and contribute to several chronic diseases [129]. The inflammation had been identified by swelling, pain, loss of tissue function, redness [130]. The triggering event during the inflammation process includes leukocyte accumulation and recruitment and vascular permeability [128–131]. Various factors cause inflammation that can be non-infectious and infectious that triggered cytokine production at the site of inflammation [132].

2.6.1 Apoptotic Pathway

For maintaining the whole function of an organism millions of cells die and proliferation every day which had been very essential for tissue homeostasis and regulation of normal growth. If the cell normal growth and death imbalances disease will happen e.g., acute pathogenesis (stroke, liver failure, heart failure), diabetes, cancer, and neurodegenerative disorder. Some neurodegenerative diseases were linked with Huntington, Parkinson's disease, Alzheimer's disease that shows the phenomena of mitochondrial dysfunction, ER dyshomeostasis, and unfolded protein created. In these cases, some neural brain cells damage and shows apoptosis.

The caspase-independent apoptosis happens due to the release of ROS and Ca+2 that causes AIF release and nuclear translocation [96, 112, 127]. The Apoptosis was accompanied by increase cell membrane wrinkled, cytosol, Ca+2 release, and DNA fragmentation. Some of the key proteins which contribute to this process were caspase-9, caspase-3, caspase-8, BID, BCL2, BAX [8]. It was the form of programmed cell death that was essential for normal development e.g., removing unnecessary cells during morphogenesis and disposing of unreactive cells whose dysfunction leads to cancer. The intrinsic apoptosis was triggered by stress signals which include DNA damage, Oncogenic activation, UV radiation, growth factors. The DNA damage activates P53of BH3 protein that neutralizes BAX, BID, Anti-apoptotic proteins. Furthermore, the formation of BAX and BID activates mitochondrial transmembrane activity and cyto C efflux induces oligomerization APAF1 which activates apoptosome from caspase -9 that further activates caspase 3 and caspase 7. The extrinsic occurred through the death receptor that activates caspase-8, caspase3,caspase7. The caspase 8 activation leads to BID and BAX that activate the intrinsic pro-apoptotic protein. The DNA damaging agents induced intrinsic apoptotic genes by the release of cystic that activated caspase 9, caspase3, and caspase 7. The cystine protease caused the demolition of apoptosis by IAPs proteins that inhibit caspase activity [112, 127].

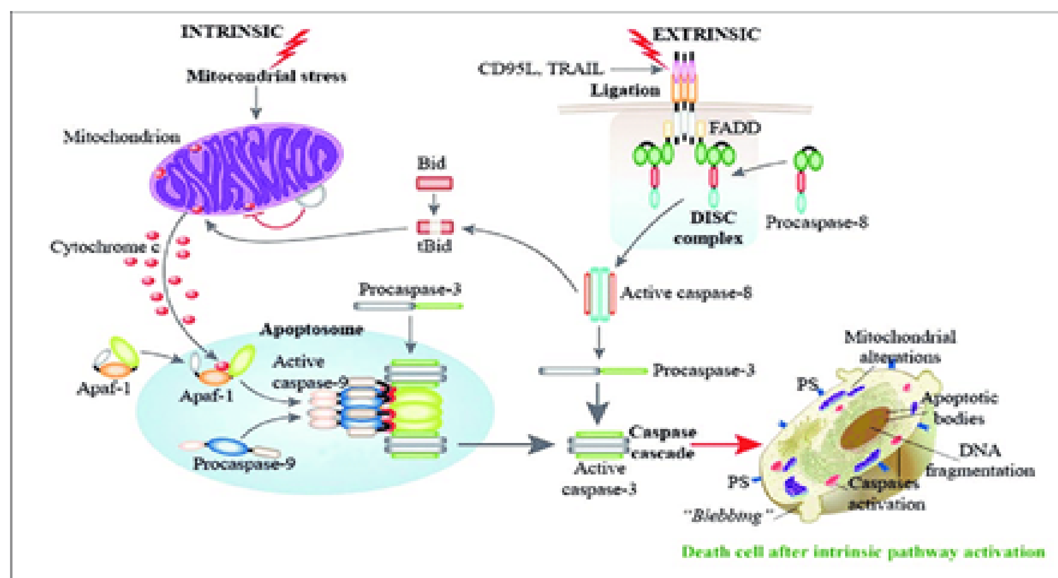

Figure 2.3: Apoptosis: The intrinsic process required stress signals that had been produced from DNA damage by p53 activation of BH3 [112, 127]

2.6.2 Apoptosis and Associated Diseases

In the case of cancerous cells, the several apoptotic bodies did not release cytosol and will not lead to fatal inflammation, in this way patient will have the least side effect to fight against diseases. The figure 2.4 shows the role of apoptosis in autoimmune diseases. The inflammation-associated apoptosis occurs in the cell that is related to the caspase family. The tissue may exist in apoptotic form and provided an immune reaction by forming inflammation. In this regeared, if apoptosis had not happened the proliferation and inflammation will occur in cancerous cells that were bad for organisms [112, 127, 133].

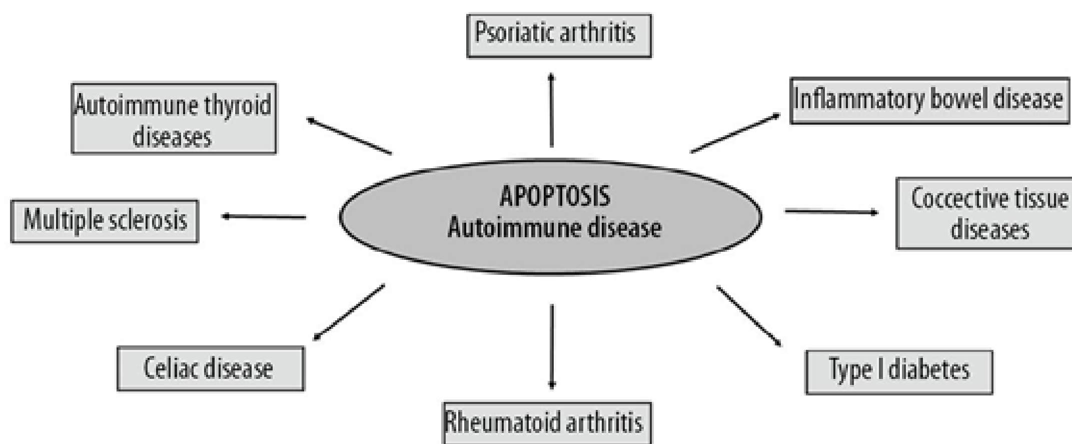

FIGURE 2.4: Apoptosis and auto-immune diseases [101]

2.6.3 Inflammatory Pathway

The inflammatory pathways affect the pathogenesis of many chronic diseases and regulatory pathways that are common mediators. The inflammatory stimuli trigger intracellular signaling pathways which had been involved in the production of inflammatory mediators. The primary inflammatory stimuli cytokines and microbial products such as interleukin-1B(IL-1B). Tumor Necrosis Factor a(TNF-a), Interleukin-6 (IL-6) mediates the process by interaction with IL-1 receptor (IL-1R), IL-6 Receptor (IL-6R), TNF receptor (TNFR). The receptor activation triggers the pathway which was involved in intracellular signaling like NFKB (Nuclear Factor Kappa B), Janu Kinase(JAK), Mitogen-Activated Protein Kinase (MAPK), Signal transduction, and activator of transcription (STAT) pathways [134, 135].

The normal pathogenic processes were distinguished by the access and response of therapeutic markers that were used in clinical application. Inflammatory markers may be correlated with the cause and consequence of several inflammatory diseases such as infection, vascular endothelial, cardiovascular diseases [136]. The inflammatory response was generated by interrelated network of cell types. The activated monocyte, macrophage, and other cells initiate the response to tissues and infection damage. The inflammatory cascade was triggered at the site of infection by the damaged endothelial cells along with some cytokines and chemokines which attract monocytes and neutrophils. The first cells attract at injuries site were neutrophils by following monocyte B, T, NK, and mast cells. Monocyte differentiates into dendritic cells and macrophages which are recircuited at the site of tissue injury [137].

The neutrophils also damage the host cells and tissues along with targeting microorganism' key mediators of the inflammatory response were neutrophils and antigen-presenting cells that activate T cells and release factor to dendritic cells and monocytes. Macrophages were crucial for maintenance, injury resolution, inflammation which had been the important component of the mononuclear phagocyte system. The macrophages present antigen, undergo phagocytes along with modeling immune response through the production of growth factors and cytokines. The activated mast cells release inflammatory mediators which include chemokines, leukotriene, cytokines, histamine, and serglycin [138].

2.6.4 Organ-specific Inflammatory Responses

15% of human cancer had been associated with inflammation and chronic infection. Acute and chronic inflammation mediate tissue injury, which was observed in many organs like the brain, reproductive systems, heart, liver, intestinal tract, lung, pancreas, kidneys [51]

2.6.4.1 Heart

The pathology of hear diseases, arteriosclerosis was the major reason for disability and death [139]. Atherosclerosis had been caused by inflammatory mediators from the recruitment of initial leukocytes to rupture atherosclerotic plaque. The early

event of cardiac stress was inflammation. The cardiac tissues elevated the level of endothelial adhesion molecule, chemokine release, and production and increased inflammatory cytokines [140].

2.6.4.2 Pancreas

The inflammatory disease of the pancreas was pancreatitis that had been caused by trypsinogen gene mutation, alcoholism, and pancreatic duct obstruction. It was characterized by the activation of inflammatory cells like neutrophils, granulocytes, macrophages, and cell destruction. The cytokine promotes CP by pancreatic stellate cells (PSCs) [141, 142]. Pancreatitis development had been required for various pathways like MAPK, JAK-STAT, NFKB that cause cell activation in the inflammatory area [140].

2.6.4.3 Liver

Normally inflammation protects the liver from injury and infection, but excessive inflammation leads to loss of hepatocyte, metabolic alteration, ischemia-reperfusion injury, permanent hepatic damage eventually. Inflammation increased the chance of chronic liver disease also destroys hepatic parenchymal cells [51].

2.6.4.4 Lung

The complex interaction between structural and immune cells causes inflammatory disease [51]. The inflammation in the lung had been caused by bacterial, viral pathogen exposure from the environment. Excessive acute inflammation and lung injury caused the impaired gas exchange and pulmonary fibrosis. Furthermore, unresolved lung injury and chronic inflammation were observed in asthma, chronic obstructive pulmonary disease (COPD). 90% of COPD had been caused by smoking-induced inflammation in the lungs and small airways. Cigarette smoke was the major risk factor for (COPD) which had been involved in pulmonary and systematic inflammation [111].

2.6.4.5 Kidney

The progressive renal injury was caused by inflammation in the kidney which leads to chronic kidney disease (CKD), end-stage renal disease, glomerulonephritis [137].

Almost 50% of older patients show signs and symptoms of kidney dysfunction and 10-12% of the population suffer from CKD which had been related with high mortality and morbidity. The CKD and Acute kidney injury(AKI) was a severe type of injury [137].

2.6.4.6 Intestinal Tract

Acute and chronic inflammatory disease of the intestine can leads to health disturbance [143]. IBD includes chronic disease (CD), Ulcerative colitis but also nonlethal inflammation of the bowel. The IBD appears to be dysfunctional interaction between the mucosal immune system and gut bacteria [140].

2.6.4.7 Reproductive System

The normal reproductive system was also affected by hallmarks of inflammation including menstruation, ovulation, and patriation. The healing and injury caused by ovulation and menstruation had been associated with the initiation of the inflammatory cascade. The maintenance and initiation of the inflammatory process had been associated with the reproductive tract [144].

2.6.4.8 Brain

In the brain, the inflammatory response leads to autoimmune disease, epilepsy, Parkinson's disease, Alzheimer's disease, etc. The inflammation in the brain leads to injury cells, neural accessibility, blood-brain permeability to the vascular molecule. The inflammation-associated CN disease produces pro-inflammatory markers from the activation of brain resident immune cells [140].

2.7 Modeling of Biological Network

A large amount of immediate data regarding molecular aspects of biological processes provided the outcome that living organisms expressed the complex systems, that levelheaded the dense interacted network. The structure and functionality of the system would be provided by the network. The new methods were developed that played a significant role in modeling biological systems. For this, the formal models studied the systems by using the language of mathematical theory [4].

The modeling and simulation play a crucial role as they provide a system-level understanding of the biological system by explaining the behavior of the system. The behavior of the network could be affected by randomness which arises from intrinsic noise, external environment, and lower number of molecules. Randomness was an inherited property that had been induced by the metabolic network, gene frequency network, and signal transduction [28]. Different approaches for modeling biological systems were provided one of them was the Petri net theory that had been provided by Carl A. in computer systems [5], In the mid-1990s they were applied for analysis and modeling of biological systems [26]. The Petri net provided modeling and simulation of biological systems through the development of intuitive graphics and several tools. Stochastic modeling deals with randomness. stochastic differential equations, stochastic Petri net (SPN), stochastic process algebra [46, 139]. As compared to the deterministic approach the stochastic approach had been more accurate than ODE. The SPN was a promising tool for analyzing and modeling stochastic biological systems [145, 146].

Sarmady et al performed system-based computational analysis by using experimental data from specific molecular interactions to identify molecules that play a significant role in the pathogenesis of Human Immuno Viruses (HIV). The study provided the dynamic behavior for the discovery of motif discovery algorithm on the sequence of immediate binding protein partners found on the host with a specific group of HIV viral proteins by providing the conserved viral sequence [147]. The dynamic behavior was predicted and constructed from the behavior of a group of elements through quantitative measurement through computational or mathematical models. The graphical models provided the mechanisms behind the experimental systems. These models provide the static picture biological system that was simulated by using a computational programmer. With the tight collaboration between biologists and computer scientists, the researcher provides information that both systems share common features. Just like computational systems the biological systems also consist of various components whose behavior was predicted by the interaction of those components. For this analysis and specification, the formal techniques were designed for developing the biological domain.

Developing biological domain-specific methods and computational systems by successful application of these techniques to biological systems formal and executable models offers an excellent means to present knowledge about biological systems and the reason behind these systems [148].

Furthermore, modeling and simulation play a crucial role as they provide a system-level understanding of the biological system by explaining the behavior of the system. The randomness was an inherited property that had been induced by the metabolic network, gene frequency network, and signal [147]. Traditional In vivo systems were expensive and take time. However formal models take less time and effort for In-silico's evaluation of the hypothesis. It only required the initial assignment of parameters and variables. Table 2.3 provides a list of widely used models and their specifications.

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TABLE 2.3: A list of widely used tools for Modeling

		Modeling language/approach/technique											**Execution**			**Formal Analysis**				**Ref.**
No.	**Tools**	**Agent-based**	**Boolean/Qualitative**	**Biochemical**	**Compartment based**	**Hybrid system**	**Lattice-based**	**Markov chain**	**Petri net**	**Process algebra**	**Rule base**	**Stat chat**	**Continuous semantics**	**Discrete/Boolean semantics**	**Stochastic semantics**	**Model-checking**	**Parameter synthesis**	**Runtime verification**	**Static analysis**	
1	BAM	–	–	–	√	–	–	–	–	√	√	–	–	–	√	–	–	–	√	[146]
2	BIOCHAM	–	–	–	–	–	–	–	–	–	–	–	√	√	√	√	√	√	√	[149]
3	REPLAST	√	–	–	–	–	–	–	–	–	–	–	–	√	–	–	–	–	–	[150]
4	S–TALIRO	–	–	–	–	√	–	–	–	–	–	–	√	–	√	–	√	√	–	[151]
5	SPIM	–	–	–	–	–	–	–	–	√	–	–	–	–	√	–	–	–	–	[152]
6	SNOOPY+MARIE	–	–	–	–	–	–	–	√	–	–	–	√	√	√	√	–	–	–	[146, 153]
7	ROVERGENE	–	–	–	–	√	–	–	–	–	–	–	–	√	–	√	√	–	–	[145]
8	PRISM	–	–	–	–	–	–	√	–	–	–	–	–	–	–	√	√	–	–	[114]

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9	PATHWAY LOGIC	–	–	–	–	–	–	–	√	–	–	–	–	√	–	√	–	–	–	[154]
10	GINSIM	–	√	–	–	–	–	–	–	–	–	–	–	√	–	√	–	–	–	[139]
11	FLAME	√	–	–	–	–	–	–	–	–	–	–	–	–	√	–	–	–	–	[155]
12	BLODIVINE	–	–	√	–	√	–	√	–	–	–	–	√	√	√	√	√	–	√	[156]
13	BNS	–	√	–	–	–	–	–	–	–	–	–	–	–	–	√	–	–	–	[157]

2.8 Molecular Docking

The modeling and simulation play a crucial role as they provide a system-level understanding of the biological system by explaining the behavior of the system. The behavior of the network could be affected by randomness which arises from intrinsic noise, external environment, and lower number of molecules. Randomness was an inherited property that had been induced by the metabolic network, gene frequency network, and signal transduction. Different approaches for modeling biological systems were provided one of them was the Petri net theory that had been provided by Carl A. in computer systems, In the mid-1990s they were applied for analysis and modeling of biological systems. The Petri net provided modeling and simulation of biological systems through the development of intuitive graphics and several tools.

The advancement in procedures obtained from human genome projects like crystallography, protein structure prediction, protein purification, and NMR provided information about structural components of protein with ligands. Molecular docking is also referred to as Computer-Aided Drug Design (CADD). There are two methods to performed docking structure-based and ligand-based. The ligand based-CADD provided knowledge of inactive and active molecules by the QSAR approach whereas the structure-based technique of docking calculates interaction energy of compounds based on molecular structure. The structure-based QASR was selected when information about ligand was large otherwise structure-based method was employed [158].
Several tools had been developed and utilized for computational analysis of ligand and protein to identify good and small structural drug molecules. The tools included Glide, ICM, FITTED, Gold, PyRX, MOE, Prix. The MOE(Molecular Operating Environment) enables to characterize, evaluate and visualize the interaction of the protein with ligands. The in-silico design applications and modern Structure-Activity Relationship(SAR) enables the design of micro molecule or ligand for protein structures. It operates on information technology, energy determination, high throughput screening, and docking [159]. Table 2.4 provided a list of widely used tools for docking and their advantage.

Table 2.4: A list of widely used tools for docking

No	Tools	Algorithms	Scoring term	Advantage	References
1	Molecular Operating Environment (MOE)	Alpha shapes and high-speed algorithm	Flex X, London dG, Drug score, Mc dock	Available score code shows the position of interacting amino acid, customizable, provide binding affinity	[160]
2	Prix	Lamarckian genetic algorithm	Internal energy binding affinity	Excellence thermal property at high and low temperature is its key feature	[161]
3	Autodock	Lamarckian generic algorithm	The empirical free energy function	Adaptable to user-defined input	[162]
4	Glide (grid base ligand docking with energy)	Manto-Carlo	Glide score	Lead optimization and lead discovery	[163]
5	Surflex	Surflex-Dock search algorithm	Bohms scoring function	By extending force field provides high accuracy level	[164]
6	GOLD (genetic optimization for ligand docking)	Genetic algorithm	User defined, Gold score, CHEMPLP (Piecewise linear potential). chemscore	Allow atomic overlapping between ligand and protein	[165]

No	Tools	Algorithms	Scoring term	Advantage	References
7	GEMDOCK	Genetic algorithm	Empirical scoring function	Provides a graphical integral amino acid for virtual screening ,structure base integration of post and virtual screening analysis	[166]
8	FITTED (flexibility induced through target)	Genetic algorithm	Drug score and potential of mean force(PMF)	Analyze the effect of water on ligand and protein complex	[159]
9	GlamDock	Monto Carlo Method	Chillscore	Through protein targeting it provide screening of ligand by 2-D analysis	[167]
10	Fred (Fast rigid exhausted docking)	Exhaustive search engine	Garrison scoring function	Within protein active site examine all posses by non-stochastic approach	[168]
11	Flex X	Incremental reconstruction	Modified Bhom scoring functions	Provide large number of conformations	[169]
12	MVD (Molegro virtual docker)	Evolutionary algorithm	Mol Dock score	Predict the binding mode with high accuracy	[160]

No	Tools	Algorithms	Scoring term	Advantage	References
13	Ligand fit	Monto Carlo Method	PLP, Ligscore, PMP	Based on Ligscore generate good hit rate	[170]
14	ICM(Internal coordinate modeling)	Monto Carlo Method	Virtual library screening	Find parallel arrangement of two rigid helix by allowing side chain flexibility	[171]

Chapter 3

Methodology

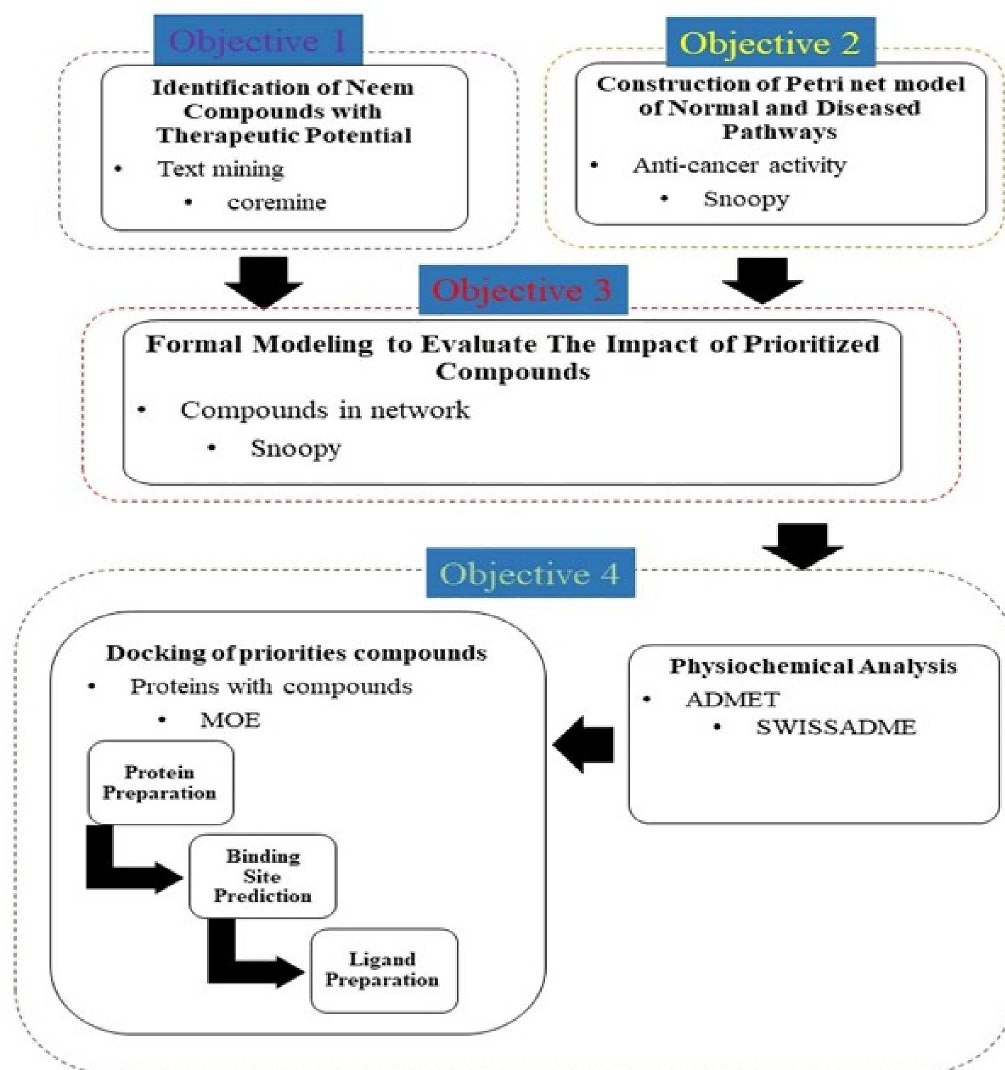

FIGURE 3.1: Steps performed to analyze and model anti-cancerous activity of *A. indica* compounds

The first objective was achieved by using text mining to identify the anti-cancerous pathways targeted by *A. indica* compounds. The identified pathways were retrieved and used to extract the interacting entities (Genes/ Proteins) needed for the construction of Petri Nets. The second objective was attained by using entities as input for modeling normal and diseased pathways through Petri nets to predict the behavior of the system. That could be used further to check the individual and combined effect of *A. indica* compounds that accomplished the third objective. This would be helpful to investigate the molecular basis of interacting entities to identify potential drug targets through physiochemical analysis and docking analysis that fulfilled the forth objective. Figure 3.1 illustrates the complete steps of the methodology. The pathways targeted by *A. indica* compounds were identified through the Coremine tool. The Snoopy tool was used to model and simulate time-dependent behavior of normal and diseased pathways that provide interacting entities which would be used further to determine the cumulative effect of *A. indica* compounds. After that swissADME was used to determine drug likeliness property that helped in drug discovery through docking by Molecular Operating Environment (MOE).

3.1 Identification of Neem Compounds with Therapeutic Potential

The number of the compounds that belong to *A. indica* have been reported to be effective against the number of cancer types that include gastrointestinal, hematological breast, skin, connective tissue cancer. So, identification of compounds that modulate cancer signaling pathways would be the first step before identifying their cumulative effect. The *A. indica* comprises almost 300 naturally products. The data on Azadirachta indica targeted anti-cancer pathways was retrieved by using text mining through the Coremine tool. It is a bio-medical text mining tool that provided information about proteins, genes relevant to MesH terms, diseases, processes, drugs, etc. from published data [6]. The papers of *A. indica* that were published in PubMed was searched by using term "azardirachta" and "anti-cancer activity". The published information was refined from database by selecting given

terms "azardirachta [MeSH term] indica" AND "anti-cancer activity" by using the advanced search option.

3.2 Petri Net Approach

The modeling of normal and disease pathways was done by using the Petri-Net(PN) approach. Petri's net theory was provided by Carl A in 1962 in computer systems [5]. Petri nets were originally constructed to represent concurrent, discrete processes of technical systems. It combines unambiguous, intuitive, qualitative bipartite graphical representation of inconsistent processes through formal representation by offering the simple and flexible language. Petri nets were also used to represent chemical reactions, signal transductions, gene expression, and neural processes by reconstruction of complex biological networks. For biological systems, the construction of the PN model involves two steps; modeling(building the model structure which represents the interaction of the built system of biochemical reactions) and simulations (specifications of parameters of transition that give the rate of biological reaction) however, the latter one is more challenging [172].

Petri nets may represent kinetics (continuous) and stochastic (discrete) processes at the subjective resolution of kinetic details by different locations in different cellular components and different localizations in 1, 2, 3-dimensional space and translocations with different locations by representing the signaling state of networks, circuits or single molecule or physiological response of cell [7, 8]. It had been practiced biological case studies like lac operon regulation, yeast cycle [9, 15]. The S.cervisiea copulatory hormones response, Duchenne muscular dystrophy [16]. It also represented the metabolic systems of the iron homeostasis process in the human body and the sucrose breakdown pathways in potato tuber [17]. The level of model abstraction can vary from single cells to multicellular aggregations even the complete population of an organism can be constructed by the Petri net approach [172].

Petri nets act as an umbrella to incorporate both qualitative and quantitative modeling under which many different analysis techniques come. Petri net allows

the validation of models like checking a model by using linear algebra, graph theory, and simulation techniques. It results in the application of simulation studies in exploring time-dependent dynamic behavior, state-space model, and analysis of structure criteria [18]. The places were passive states indicated in the circle. In the biological context, places represent organisms, species, molecules, genes, ions. The token represents the discrete values that would be consumed by transitions assign to places that affect the behavior of places within the system. In a biological context, they represent the concentration of genes, ions, and proteins. The square represents the initial active nodes. It describes the system events or activation in a network. In a biological context, the transition represents the intermolecular change, chemical reaction, or molecular change. The arcs represent the relationship between places and transition. In a biological context, they represent that either a given entity was activated or inhibited in a system provided in Figure 3.2. If the place is related to transition by arc, the place transition is called pre-place (post-transition). If a transition is connected by an arc with the place, the transition (place) is called pre-transition (post-place). The transition consumed the pre-place token and produced post place according to arc weightage.

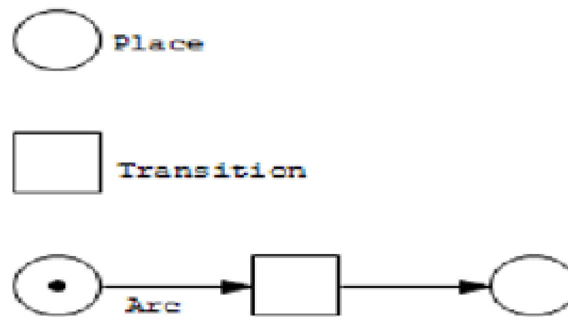

FIGURE 3.2: Graphical depiction of Arc, Places and transitions

The hybrid and qualitative Petri net model offer required knowledge of steady states, cycles as well as the connection between the rate of degradation and synthesis within entities. The Petri net provided a good executable numerical graph that may be used to research the properties of a system having interacting entities. The Stochastic Petri net was used in this study Snoopy tool was used to [155, 173] used for the modeling and simulation of the biological system. There are

several features provided by this tool Extensible (The generic design facilitates the new pertinent class), Adaptive (The graphical user interface in the active window adopts dynamically), Platform independent (Mac, Linux, Window). In a single frame, various classes of Petri-net were defined for extrinsic/intrinsic/receptors. The Hybrid Petri net (HPN) which was selected provides both stochastic and deterministic approaches for modeling the disease and normal pathways.

FIGURE 3.3: The user interface of Hybrid Petri net

3.3 Construction of Petri Net Model of Normal and Diseased Pathways

The anti-cancer activity of *A. indica* Phyto-compounds has been the subject of active research as it plays a major role in the treatment of cancer. Thus, for the modeling of any system first, we must identify the regulatory entities that trigger these processes along with their relationship in given pathways. The pathways were obtained from the data obtained from online databases; Reactome {https://reactome.org/} & KEGG {https://www.genome.jp/kegg/pathway.html}. Modeling the normal and diseased pathways the places were used to represent genes/proteins/compounds whereas transitions represent the response and arc represent the relationship that could be inhibition or activation of places. The availability of concentration on the places was represented by token whose initial

concentration two was given to ligands/foreign particles/DNA damage which triggers the given process of apoptosis and inflammation in normal and disease model whose criteria of selection was discussed in figure 3.4 that was provided by Thomas K [21] and flow of token represents the consumption of initial concentration.

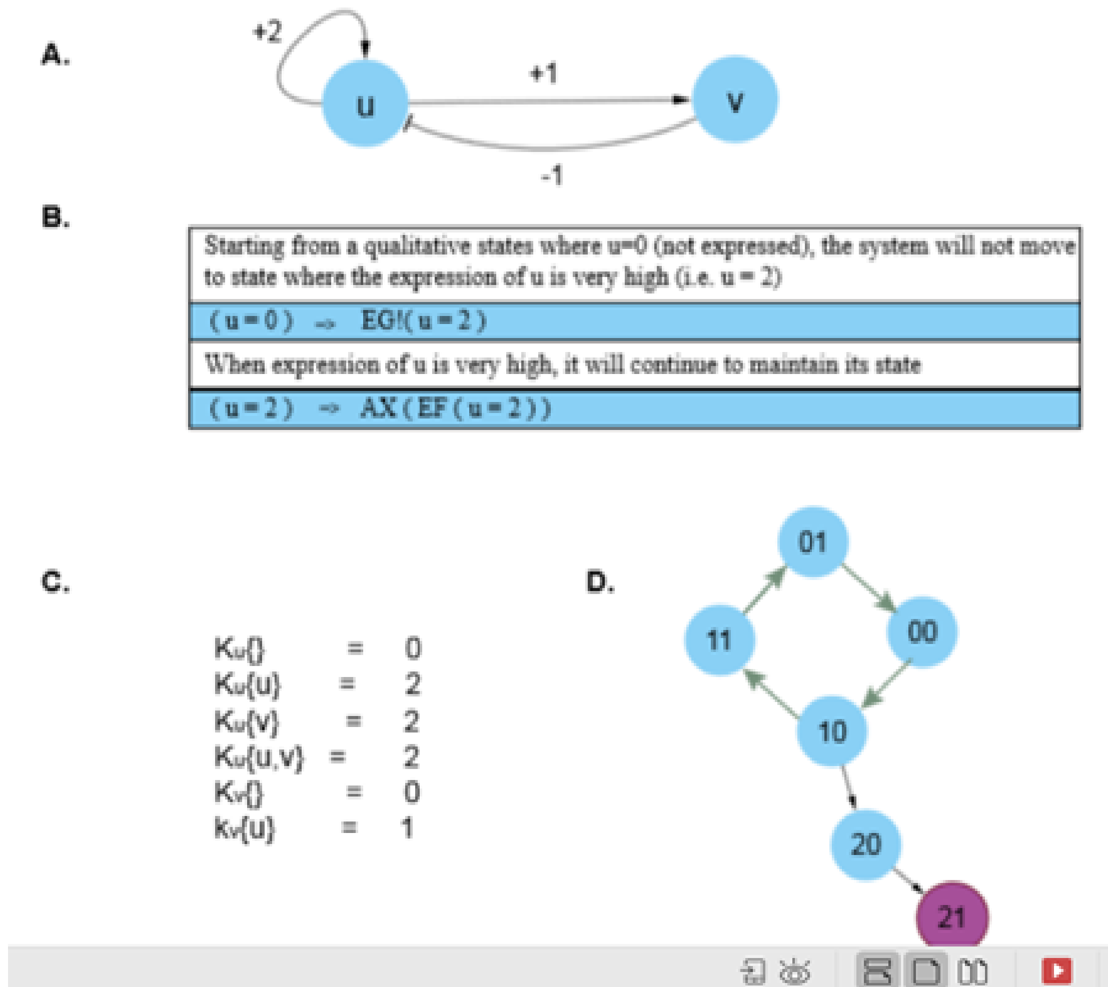

FIGURE 3.4: Represented [20] biological regulatory network of *P.aeruginosa*. (a) activation and inhibition state was provided by edges and genes by nodes that was governed by CTL(Computation Tree Logic). The network comprises two entities: *U represent ALGU and V represent its inhibitor C represent the CTL observation for combination of CTL logical parameters. The B represented the behavior of either activation or inhibition. The C represented the values required for activation and inhibition. The d represented the state graph*

Token one represents the intermediate activation of place (Gene/protein). The detail had been provided in table 3.1. For clarity and better visualization of the model, places were illustrated with different colors. To avoid overlapping of places and transitions the coarse places are indicated in blue color as shown in figure 3.5 and figure 3.6.

3.4 Formal Modeling to Evaluate the Impact of Selected Compounds

The Hybrid Petri net (HPN) was used to model the anti-cancer effect of neem compounds (Azadirone, Nimbolide, Nimbidin, Azadirachtin, Gedunin) on identified entities that were involved in diseased pathways. According to Thomas K parameters [21], the values for compounds were provided two as a token that depicted initial concentration, and given entities (gene/protein) were determined after stimulation that was executed for hundred intervals given in table 3.1 by using the Snoopy tool [19, 20]. The places were depicted with different colors;

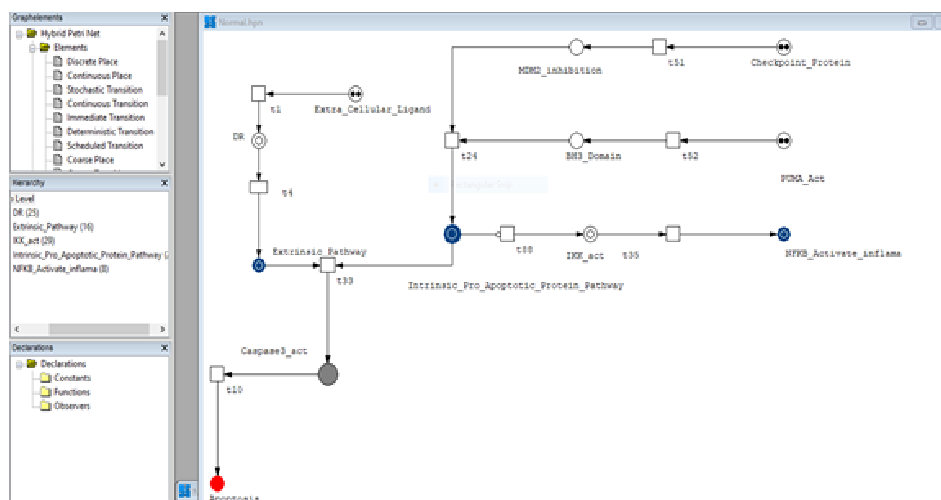

FIGURE 3.5: Model representing pathways activation in Healthy cells

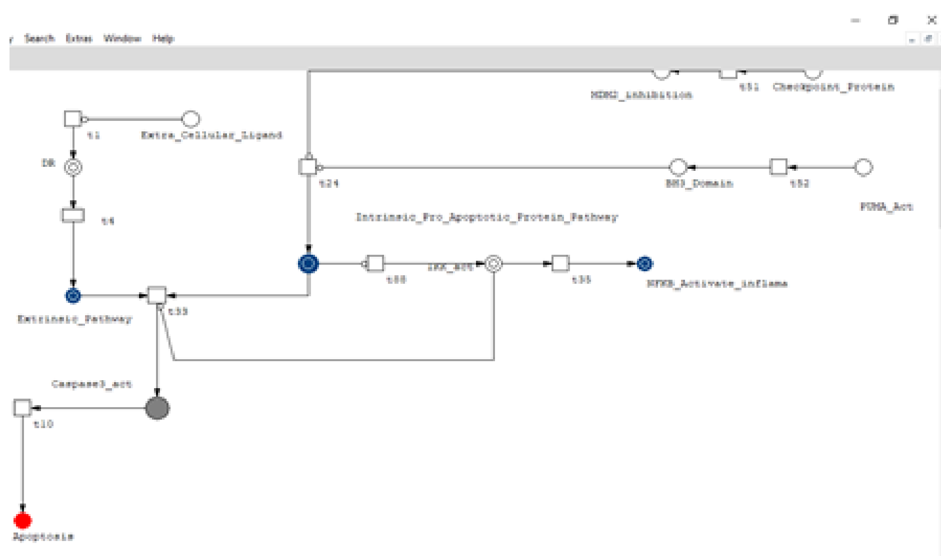

FIGURE 3.6: Model representing normal process in cancer cells

Blue color indicates the pathways which were involved in anti-cancer activity, green color indicated the *A. indica* compounds and red color represented the targeted entity figure 3.7.

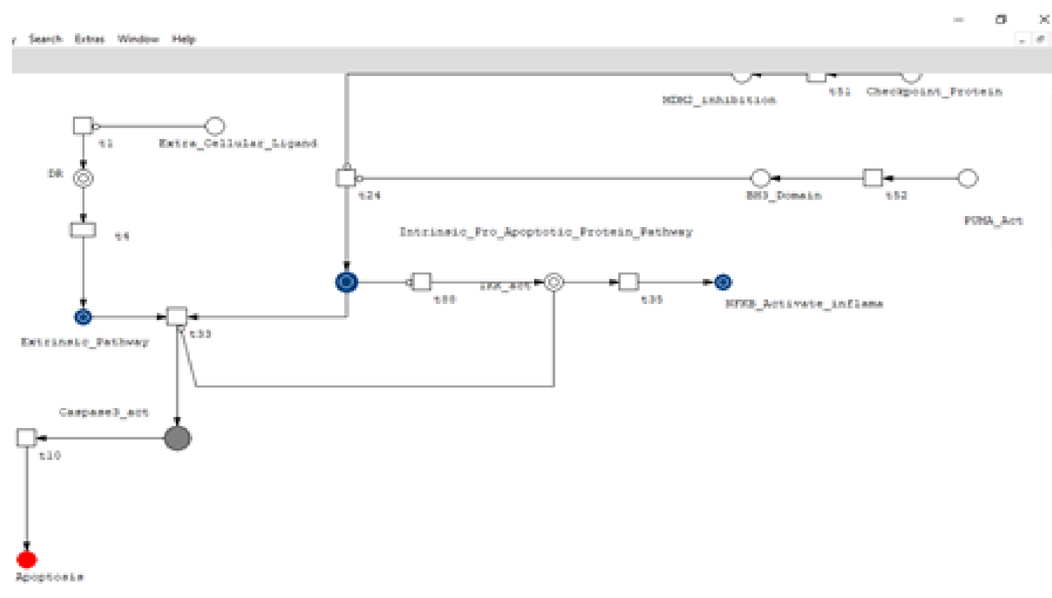

FIGURE 3.7: Model representing the anti-cancerous activity of *A. indica* compounds

TABLE 3.1: The transition of Model 3

Transition Name	Biological Event
t1	Activation by azardirone
t2	Activation of Death receptor
t3	Activation of FADD
t11	Activation of procaspase8
t13	Activation of caspase 3
t5	Activation of intrinsic process
t6a	Activation of tBid
t8a	Activation of mitochondrial transmembrane activity
t10a	Release of cytochrome C
t100	Activation of APAF1
t102a	Activation of Procaspase9
t102	Apoptosis process
t55	IKK protein inhibition

Transition Name	Biological Event
t6	Activation of IKK protein family
t41	Activation of NFKB_Inflamation by IKK
t35	Activation of NFKB_Inflamation by Nimbidin
t32	Inhibition of PEG2
E	Activation of ios_mRNA
T36	Macrophages inflammation by ios_mRNA
t34	IL1 activation
t37	Macrophage inflammation
t28	iNOS production
t27	NFKB_Activation
t24	TNF-alpha activation

Table 3.2: The transition of Model 2

Places Name	Biological Species
TRAIL	Extracellular ligand
DR	Death receptor
Intrinsic path	Intrinsic process of apoptosis
	Bid_Bax clevage
	Tbid cleavage
Bid_Bax, Tbid_clev	Mitrocondrial transmemberane activity
MTM, Cytc, APAF1,	cytochromeC
Casp9, Casp3	Apoptotic protease activating factor 1
	Caspase 9
	Caspase 3
	Process of programme cell death
	Inhibitor of nuclear factor k B
Apoptosis, IKK	Hetro trimer
P60,P50,VEGF	Angiogensis
MMP9/ICAM2	Invassion
COX/MMP9	Proliferation
IAP, BCL2_BCXL, X	Antiapoptotic genes

Places Name	Biological Species
	Anti-Apoptotic
	-
	Extrinsic process
Exrin, FADD, Proca8	FADD adoptor protein
	Procaspase 8
NFKB	Process of inflammation by nuclear factor k B
	Inducible nitric oxide synthase mRNA
Inos_mRNA,	Inflammatory Macrophages
Inflam_macro, PGE2	Prosta glandin E2
IL1, iNOS, NO, TNF	Inter Leukin 1
	Induceable nitric oxide synthase
	Nitric oxide
	Tumor necrosis factor

3.5 ADMET and Lipinski's Properties for Physiochemical analysis of Phytocompounds

The Drug likeliness properties within the human body were provided by ADMET (Adsorption, distribution, metabolism, excretion, and toxicity) of plant compounds. The Lipinski's property was provided in the term of (Molecular mass less than 500 Dalton, Molar refractivity between 40-130, Hydrogen bond acceptance less than 10, high lipophilicity (log p-value) less than 5. These values provided bioavailability of Phyto-compounds for oral consumption. The physiochemical analysis determines the adverse effects of phytocompounds that determine the violation of these rules by using the Swiss ADME http://www.swissadme.ch/ online tool [174]. The Drug likeliness properties within the human body were provided by ADMET (Adsorption, distribution, metabolism, excretion, and toxicity) of plant compounds.

3.6 Docking of Prioritized Compounds

3.6.1 Molecular Docking Procedure

Molecular docking is also referred to as computer-aided drug design (CADD). There are two methods to performed docking structure-based and ligand-based. The ligand based-CADD provided knowledge of inactive and active molecules by the QSAR approach whereas the structure-based technique of docking calculates interaction energy of compounds based on molecular structure.

TABLE 3.3: Information of Compounds and Interacted Proteins involved in cancerous pathways

Compounds	Protein Abbreviation	Protein Names	PDB ID
Azadirone	BCL2	Apoptosis regulator Bcl-2	1G5M
	BCL2L1	Bcl-2-like protein 1	2LP6
	BIRC2	Baculoviral IAP repeat containing protein 2	6ESW
	XIAP	E3 ubiquitin-protein ligase XIAP I	2LXW
	SURVIVIN	Survivin	1e31
	LRP8	Low-density lipoprotein receptor related protein 8	Icr8
Nimbin	CFLAR	CFLAR - CASP8 and FADD like apoptosis regulator	2N5R
	IL1B	IL1B - Interleukin-1 beta	5R86
	PGE2	Prostaglandin E2	6AK3
Nimbolide	BCL2	Apoptosis regulator Bcl-2	1G5M
	BCL2L1	Bcl-2-like protein 1	2LPC
	BIRC3	Baculoviral IAP repeat containing protein 3	2UVL
	PTGS2	Prostaglandin G/H synthase 2	5IKR
	CMYC	Proto-oncogene protein	5kIR

Compounds	Protein Abbreviation	Protein Names	PDB ID
Nimbolide	MMP9	Matrix metalloproteinase-9	5TH6
	ICAM2	Intercellular adhesion molecule 2	3E2M
	VEGF	Vascular endothelial growth factor A	1KAT
	BCL2	Apoptosis regulator Bcl-2	1G5M
	BCL2L1	Bcl-2-like protein 1	2LPC
Azadirachtin	BID	BH3-interacting domain death agonist	2BID
	BAX	Apoptosis regulator BAX	1F16
	P53	Cellular tumor antigen p53	6I3Y
	TCEAL1	Transcription elongation factor A like family	3NDQ

3.6.1.1 Preparation of Proteins Structure

The 3D structure of proteins was obtained from the online repository Protein Data Bank https://www.rcsb.org/.The protein structures were prepared in MOE by removing water molecules and ligands(if existed). After the removal of ligands and other atoms, the missing hydrogens were added. The energy of minimalization for structure of the was performed to get the stable conformation by preventing overlaps and saved the modified file in PDB format.

3.6.1.2 Prediction of Binding Site

The Dogsitescorer tool was used to determine the binding site or active site in protein for ligand (compound) by providing information about several amino acids involved proteins active site and drug score. Those pockets were selected having a drug score less than 1 [22].

3.6.1.3 Preparation of Ligands Structure

The ligands structure was prepared in MOE after obtaining 3D structure from chem spider or PubChem in SDF format. The ions were removed and hydrogen

atoms were added, the energy of minimalization was calculated after structure correction by using default parameters. The refined structure was saved in mol2 format.

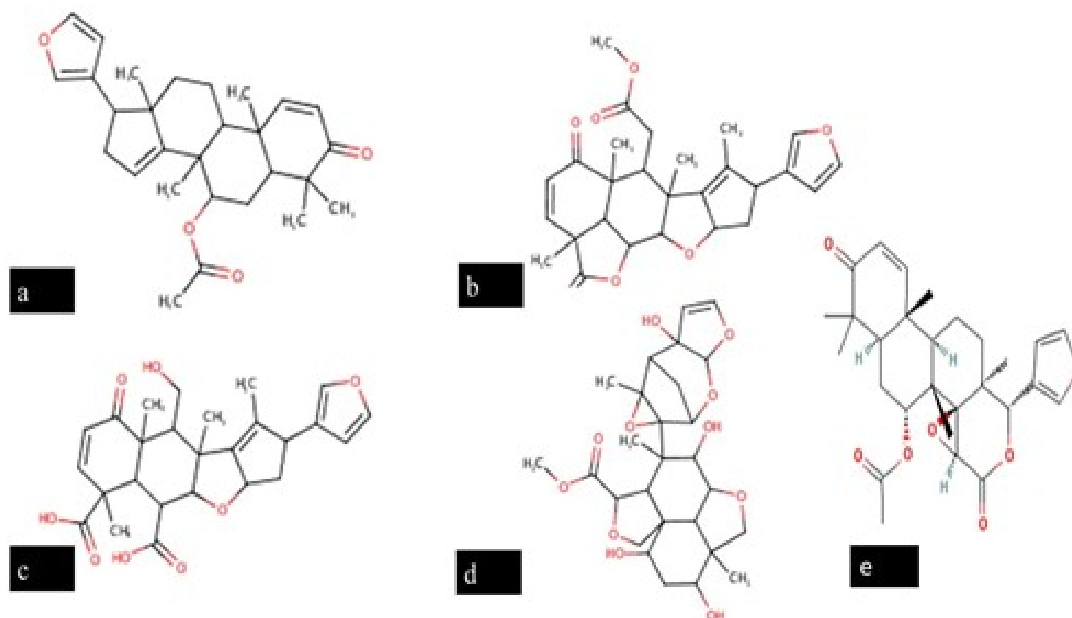

FIGURE 3.8: *Ligands structure retrived from Pubchem: Azadirone_10906239 (a), Nimbolide_100017 (b), Nimbic acid_25446 (c), Azadirachtin_183572 (d) and Gedunin_12004512 (e)*

3.6.2 Docking Process

The quality of interaction of the protein with ligand(Phyto-compound) was provided in the term of binding affinity, RMSD value and number of residues involved in binding orientation. The proteins were docked with their interacting residues in the form of ligands. For this first, the database of each ligand was created in the MDB database. The ligands were imported into the database and the structure was opened. After that, the protein file in PDB formate was imported. The docking was executed for the induced fit procedure. The 5 poses were selected for every ligand among multiple conformations based on RMSD value and binding energy [23, 24].

Chapter 4

Results and Discussion

4.1 Identification of Neem Compounds with Therapeutic Potential

A. Indica (neem) is the storehouse of various phytochemicals. The impact of 300 phyto-compounds natural products upon modern drug discovery compounds had been reported [174, 175]. There were a total of 1555 articles available for the given search term "Azadirachta indica [MeSH term]", 371 articles for "anti-cancer agent [drug]" and a total of 30 articles contain both terms in abstract or title. The five major compounds (azadirone, nimbolide, nimbidin, azadirachtin, gedunin) were selected from literature in the current study which were involved in the cancer by regulating inflammation and stimulating apoptosis.

The azardirone trigger apoptosis by signaling cancer cells to TRAIL. The TRAIL resistance involved multiple mechanisms in cancer. The down regulation of decoy receptors is the first mechanism [176, 177].The upregulation of antagonistic decoy recptor that binds with receptor but lack functional domain for iniciating apoptosis that provided second mechanism [178, 179]. The upregulation of cell survival poteins and down regulation of pro-apoptotic protein [180–184].

The multiple mechanisms of resistance may be seen for cancer. The TRAIL resistance in tumor cells was due to overexpression of cell survival protein that includes

(BCL,SURVIVIN,MCL,XIAP,IAP). The azardirone facilitates tumor sensitization by downregulating cell survival proteins that was provided in figure 4.1. It provides the first strategy in cancer therapy by overcoming TRAIL resistance [185–187] as given in figure 4.1.

Inflammation is the immune system response to harmful stimuli such as damaged cells, pathogens, irradiation, or toxic compounds [17] by initiating the healing process and removing injurious stimuli [18, 19]. Usually, during acute inflammatory responses, molecular and cellular events and interactions provide efficiently minimize the threat of infection and injury. The uncontrolled acute inflammation may become chronic and contribute to several chronic diseases [20]. The inflammation is characterized by swelling, pain, loss of tissue function, redness [21]. The triggering event during the inflammation process includes leukocyte accumulation and recruitment and vascular permeability [18–22]. Various factors cause inflammation that can be non-infectious and infectious. Certain chemicals trigger cytokines production at the site of inflammation [23].

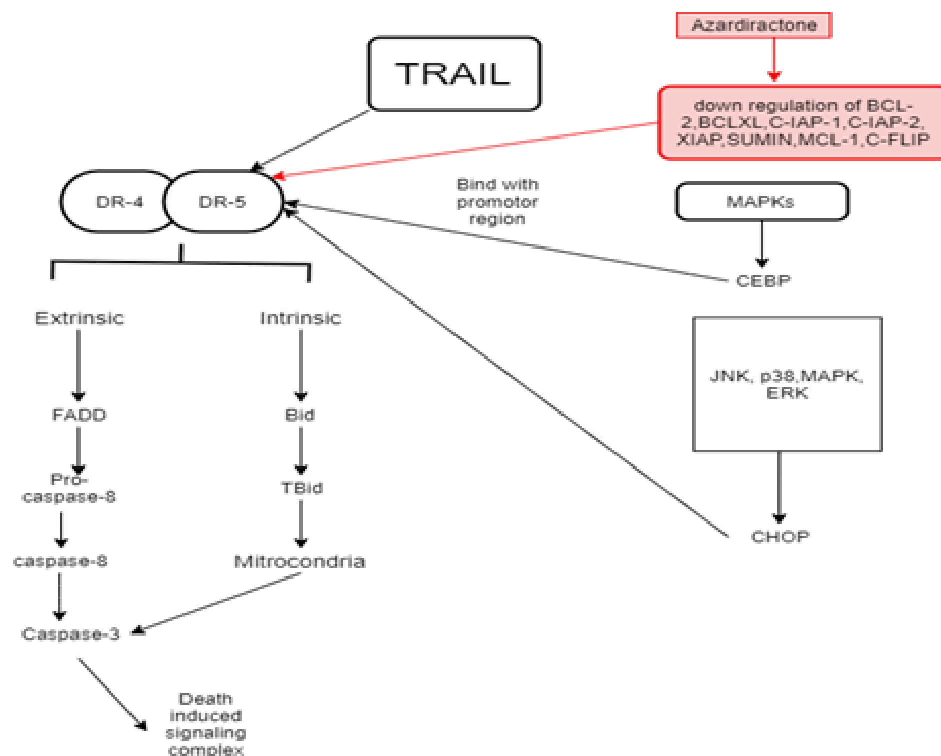

FIGURE 4.1: The diagramtic representation of azadirone shows TRAIL sensitization in cancer cells by down regulating DR5 domain that down regulate the expression of cell survival proteins [185–187]

The anti-inflammatory activity of nimbidin was provided by decreasing the expression of neutrophils and macrophages. The Nimbin contributes to inhibiting macrophage inflammation by inhibiting NO without any cytotoxic effect. This activity was contributed due to a decrease in iNOS production without affecting its enzymatic activity. It further causes iosmRNA that overall prevent inflammation by inhibiting NFKB transcriptional factor component that mediates ROS production [188]. The overall oxygen release was inhibited due to iNOS production. The NO inhibit the inflammatory response in different models [189]. The further anti-inflammatory activity was shown in figure 4.2. The nimbidin inhibit release of PGE2 that recurcuit neutrolphils with the help of certain enzymes [190]. So it not only inhibit the inflammation by preventing chemoattraction of macrophages and phagocytotic activity. This was the only identified compound that inhibit the macrophages inflammation and NFKB activation in cancer cells.

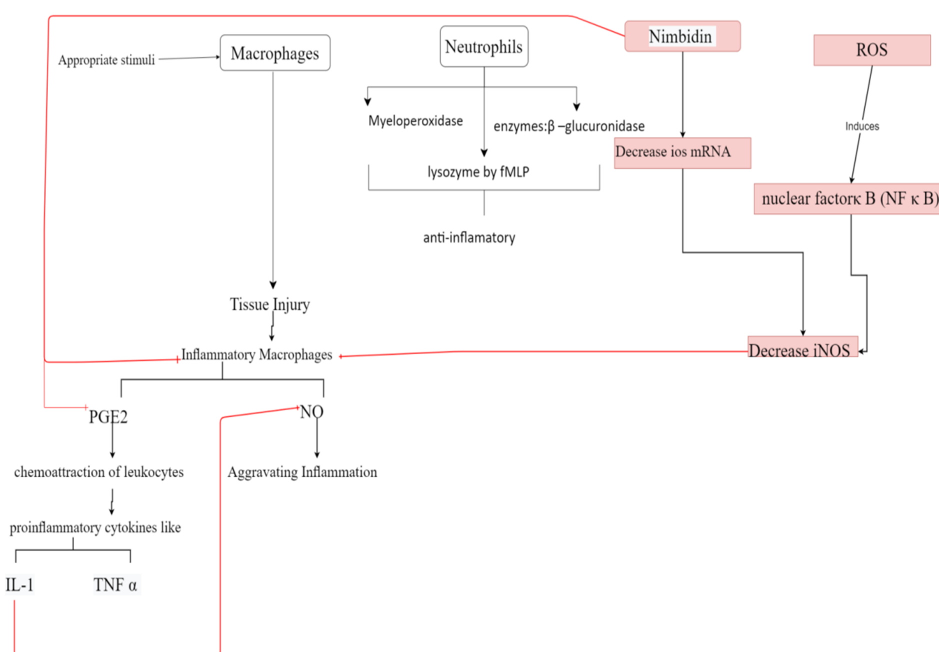

Figure 4.2: The ilustrative digram of nimbidin controlling macrophage inflammation in cancer cells by inhibiting the release of PGE2, NO, and IL-1 in cancer cells [189, 190]

The most effective activator of NFKb is TNF that plays a significant role in cell survival. The nimbolde inhibits the NFKB activation by TNF-alpha. The inhibition was performed by placing alanine at 179 position from cystine that is very crucial for the IKK domain for regulating TNF-alpha [185]. The overall impact was

provided in figure 4.3 that provided the function of NFKB was suppressed by TNF alpha that inhibiting the role of cell survival proteins (BCL, IAP), proliferation (COX), invasion (MMP), Angiogenesis(VEGF) [106]. The overexpression of these proteins causes tumor cell survival by chemoresistance or radioresistance. Hense nimbolide inhibit the function of cell survival proteins by amino acid replacement at 179 positions of IKK protein.

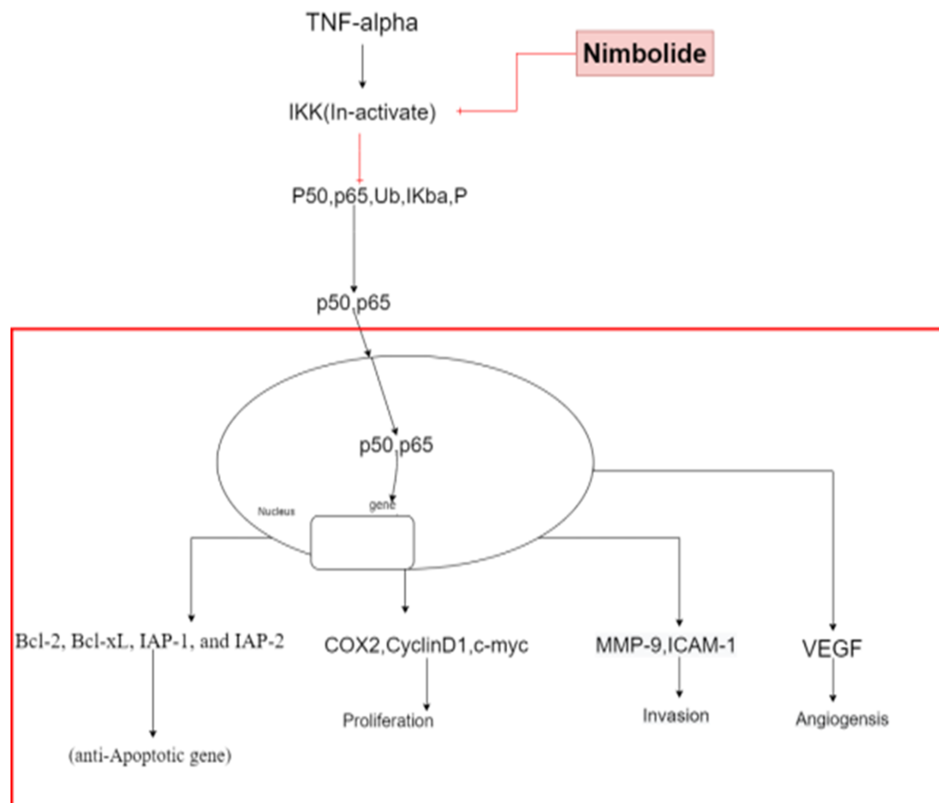

FIGURE 4.3: The diagrammatic representation of nimbolide that suppress anti-apoptotic proteins expression through IKK modulation of CYS195 with Alanine amino acid in cancer [118]

The BCL2 protein family controls the outer mitochondrial membrane permeability by stimulating cell death [6, 182]. The Bax protein causes the release of cytochrome c that was crucial for the outer membrane permeability of mitochondrial. The cytochrome c release was also enhanced by the cytosolic release of P53 that further inhibits the expression of anti-apoptotic protein [185]. The P53 also causes Bax oligomerization and is also involved in mitochondrial destabilization that causes apoptosis [191]. The combined effect of nimbolide and azadirachtin was provided

in Hela cells [185]. The two compounds cause an increase in expression of pro-apoptotic protein Bax/Bid and a decrease in the expression of cell survival protein BCL. The compounds further promote the mitochondrial transmembrane activity due to activation of p53 that controls BCL expression and cytochrome c release that was involved in apoptosis.

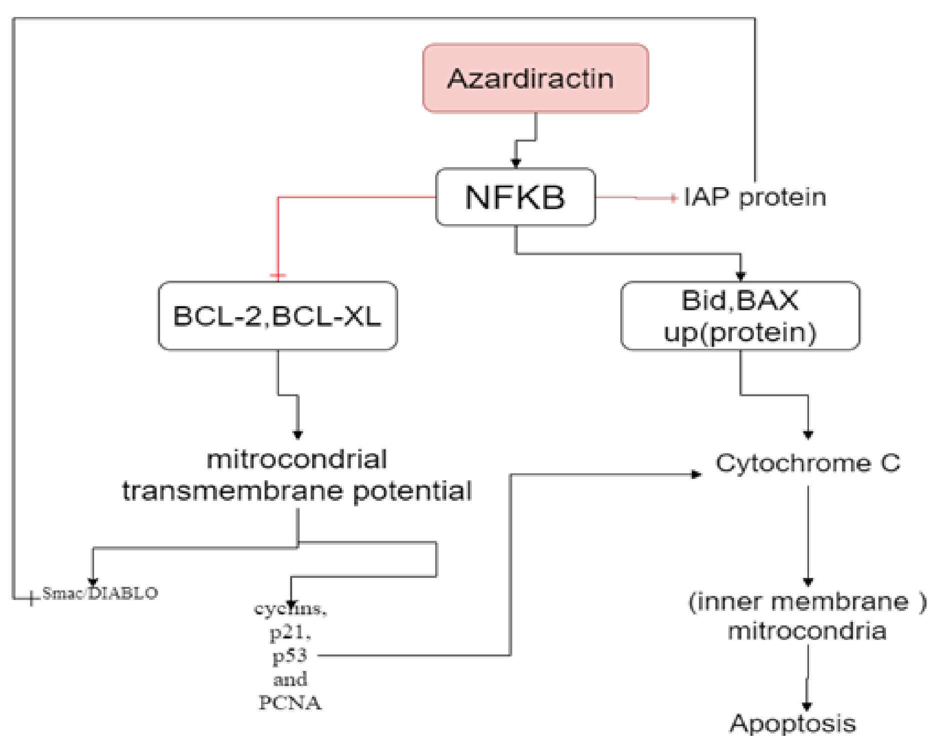

FIGURE 4.4: The ilustrative digram of azadiractin modulating apoptosis in cancer cells by inhibiting cell survival protein and enhansing the expression of pro-apoptotic proteins that help TRAIL to sensitixze cancer cells [118]

The expression of cell survival proteins was downregulated by azadirone. The overexpression of cell survival protein in cancer cells was contributed to TRAIL resistance [185, 186]. The nimbidin control the inflammatory response by ROS mediated NFKB induction through inhibitting the function of neutrophils nad macrophages [118].

The nimbolide controls the expression of cell survival proteins by replacing cysteine with alanine amino acid in TNF-alpha domain. This affects proliferation, angiogenesis, invasion, and cell survival by abrogating protein function triggered by TNF-alpha [118, 185, 191]. The azadirachtin inhibits the expression of cell survival proteins and increase pro-apoptotic protein expression in cancer [118]. In

the light of the above discussion, it was provided that apoptosis and inflammatory pathways were affected after exposure to neem compounds. Before identifying the impact of the potent compound in anti-cancer activity the interacting entities from Petri net model of normal and disease pathways were identified.

4.2 Construction of Petri Net Model of Normal and Diseased Pathways

Apoptosis is crucial for maintaining a stable internal environment by regulating certain genes that contribute to cell proliferation. In normal cells, this process is triggered either by a caspase-dependent or caspase-independent process from zero to value two. The extrinsic process requires binding of the ligand with receptor normally Death Receptor (DR). The DR is related to the Tumor necrosis factor receptor superfamily that includes TNF-related apoptosis-inducing signal, TRAIL TNF-related apoptosis-inducing ligand, TNFR1 (CD12), TRAILR1 (DR4), TRAIL-R2 (DR5), and DR6, Fas (APO1, CD95). The Fas is a well-known receptor that exists in two forms. However, for apoptosis, as given in figure 4.5, the Fasm and FasL cause death-inducing signal complex with triggers the activation of procaspase 8 and procaspase10 shown in yellow peak by activating downstream signal cascade. Furthermore, the concentration of pro-apoptotic proteins was indicated in the pink peak that triggered the caspases for apoptosis was enhanced from zero to 0.74 value. The literature study supported that there were several factors for immortality in cancer. The activation of the intrinsic process of apoptosis the UV radiation, DNA damaging agent, oncogenic activation, or growth factors were required for activating a cascade of caspases. The P53 of BH3 protein was activated by DNA damaging agent that neutralizes the Bid and Bax activation. Furthermore, the activation of caspase 3 and caspase 7 was activated from caspase 9 that was triggered by oligomerization of APAF1due to the formation of Bax and Bid [138].

On the other hand, inflammation was triggered by foreign invaders like bacteria or viruses (provided in yellow color) that activated the PEG2 from initial zero to value two (green color) which causes chemotactic attraction of cytokines. The

PEG2 further activates IL-1(red color) production that aggravates the inflammation from zero to value two (in pink peak) [138].

The literature study provided that inflammatory pathways affect the pathogenesis of many chronic diseases and regulatory pathways that are common mediators. The inflammatory stimuli activate intracellular signaling pathways which are involved in the production of inflammatory mediators. The primary inflammatory stimuli are cytokines and microbial products such as interleukin-1B (IL-1B). Tumor Necrosis Factor a (TNF-a), Interleukin-6 (IL-6) mediates the process by interaction with IL-1 receptor (IL-1R), IL-6 Receptor (IL-6R), TNF receptor (TNFR). The receptor activation triggers the pathway which is involved in intracellular singling like NFKB (Nuclear Factor Kappa B), Janu Kinase(JAK), Mitogen-Activated Protein Kinase (MAPK), Signal transduction, and activator of transcription (STAT) pathways [25, 27]. The coordinated network of cell types was involved in the inflammatory response [27, 28].

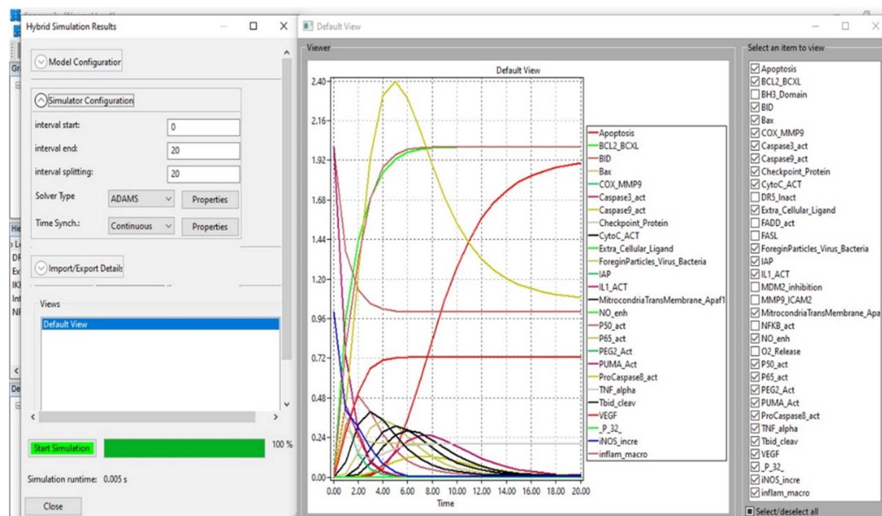

FIGURE 4.5: The normal process of apoptosis and inflammation

In the case of cancerous cells, the activation of anti-apoptotic proteins like VEGF prevents the process of apoptosis (red peak) by inhibiting mitochondrial transmembrane activity from zero to 0.2 value at the end of the simulation result. However, the inflammatory process (pink peak) was enhanced by an increase in NOS production from level zero to two (blue peak) and a decrease in oxygen release from zero level to 8.514E-8 due to PEG2 protein activation. The literature

study provided that are several hallmarks of cancer that exist in every cancer regardless of cell type or causes; these include angiogenesis, evasion to apoptosis, and uncontrolled growth like in Breast cancer, osteosarcoma, Breast cancer, Lung cancer, Melanoma cancer, Colon cancer, Glioblastoma cells, leukemia, Pancreatic cancer [29, 30]. In most cases, the intrinsic pathway is inhibited. The evasion of apoptosis could be observed either due to inhibition of caspases [13] or due to upregulation of anti-apoptotic protein BCL2 and the downregulation of pro-apoptotic protein BAX or BAK [13, 30]. In liver and prostate cancer, the level of Fas was transcriptionally down-regulated [31]. The primary and multiple levels of resistance to TRAIL-induced apoptosis [32].The p53 causes apoptosis through BCl2/Bax IGF regulation of DNA damaging genes with the ineffective repair. 50% of cancer by p53 gene and 80% are by dysfunctional of P53 signal. The upregulation of BCL2 and downregulation of Bax/PUMA was due to abnormal activation of P53 [33].

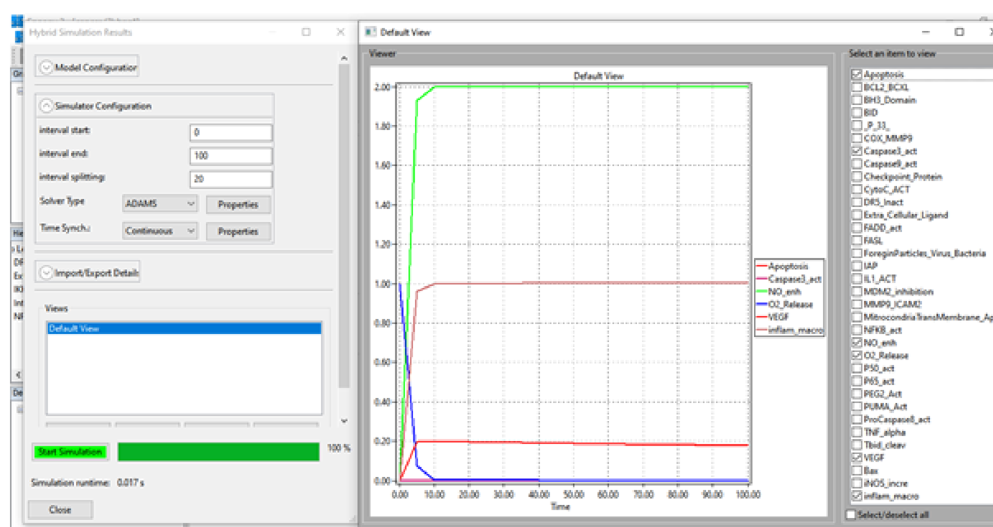

FIGURE 4.6: Apoptosis and inflammation in cancer. Table A.1 in Appendix provide detailed information on each entity after exposure to compounds

4.3 Formal Modeling to Evaluate the Impact of Selected Compounds

The cumulative effect of A.indica compounds was modeled to determine the rate of apoptosis and inflammation which were crucial for anti-cancerous activity. The azardirone upregulates the expression of the pro-apoptotic protein. It further

sensitizes tumor cells to TRAIL by modulating signaling molecules through t1 transition [152]. The azardirone exerts its anti-cancerous activity by sensitizing the tumor cells to TRAIL so altered the signal molecules that regulate apoptosis [185–187] by the rate of two as provided in Figure 4.7.

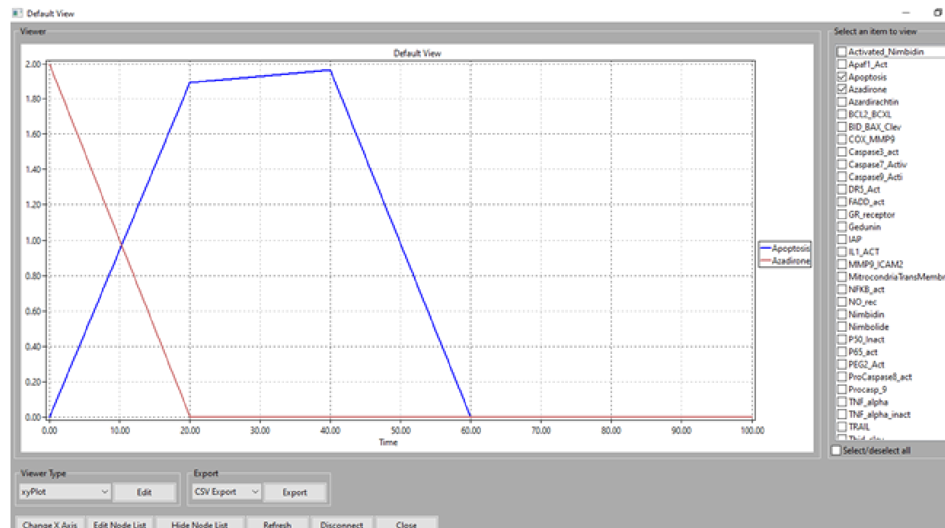

FIGURE 4.7: Individual Effect of Azardirone on apoptosis

Similarly, nimbidin inhibit the function of macrophages and neutrophils which implicit the anti-inflammatory activity by T38 transition through inhibition of PEG2 and production of iosmRNA in NFKB inflammatory pathway. This not only suppresses the phagocytotic activity but chemotactic activity as well [189]. So, it affects the anti-inflammatory activity of cancerous cells from two to zero values as provided in figure 4.8.

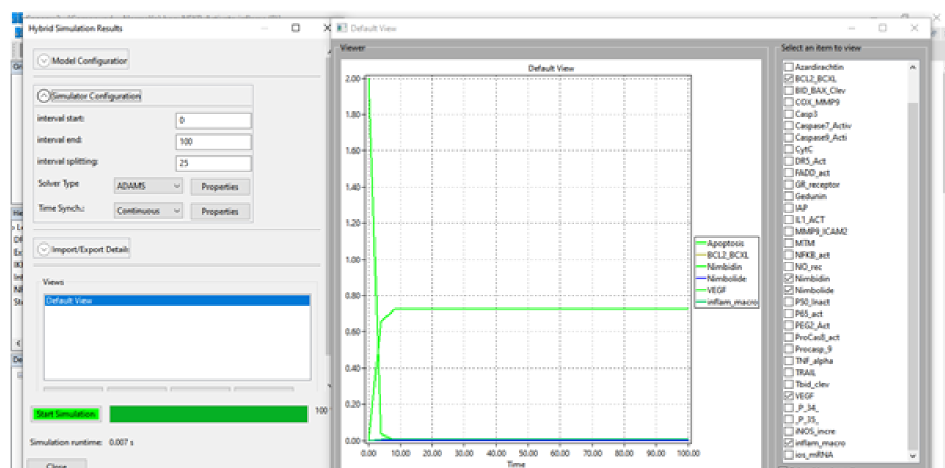

FIGURE 4.8: Result of Simulation shows the impact of Nimbidin on apoptosis regulation

Nimbolide inhibits the activity of cell survival proteins through t6 transition in the IKK process by modulating cys179 residue [185]. These proteins had been associated with NFKB inflammatory pathway and apoptosis. The individual contribution of nimbolide to apoptosis from the rate of zero to 0.1 is provided in figure 4.9.

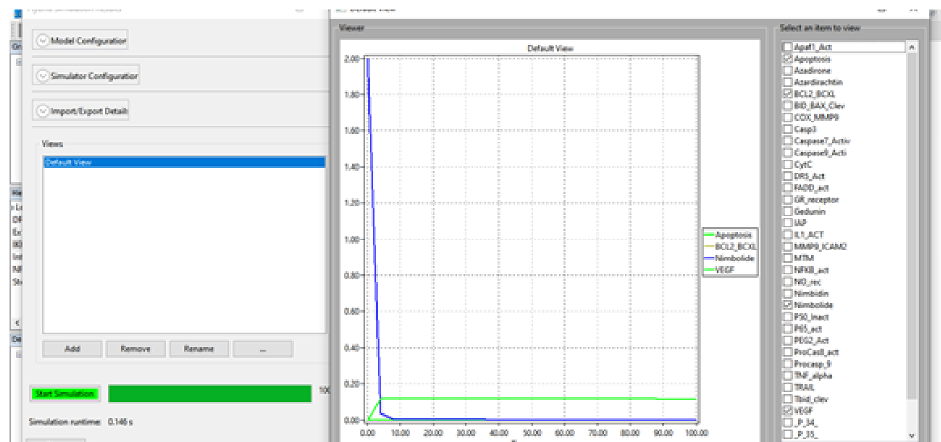

FIGURE 4.9: Simulation effect shows the impact of Nimbolide on regulating apoptosis

Similarly, azadirachtin increases the expression of pro-apoptotic protein and inhibits cell survival proteins by increasing the expression of Bax and Bid which are involved in the intrinsic process of apoptosis through t5 transition [11, 34] provided in figure 4.10 from zero to two.

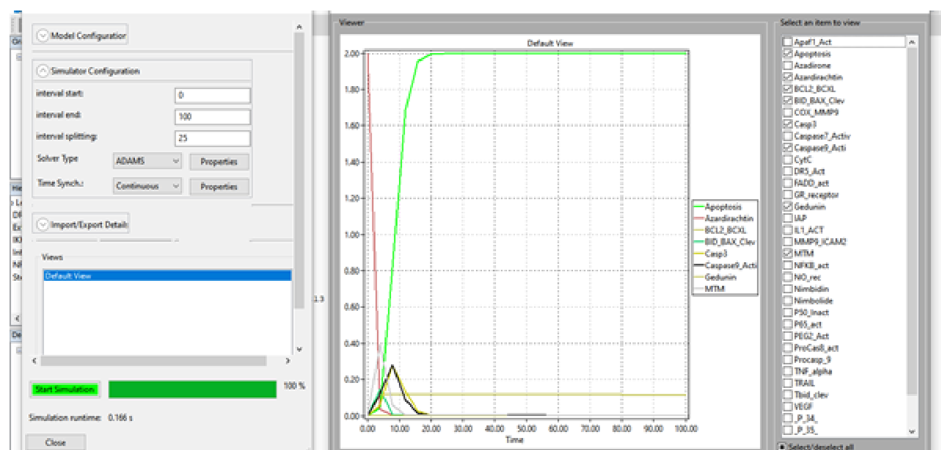

FIGURE 4.10: Simulation effect shows the impact of Azardirachtin on apoptosis regulation

The gedunin inhibits through steroid receptor by t76 transition and triggers the process of apoptosis provided in figure 4.11.

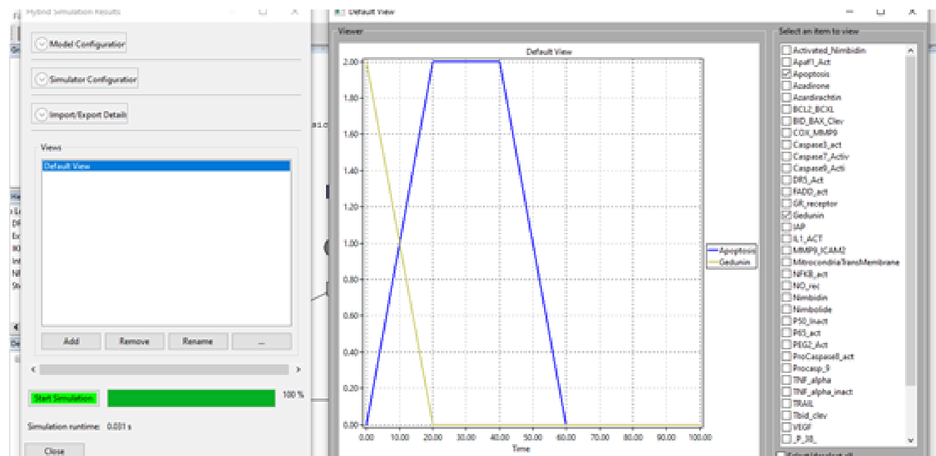

FIGURE 4.11: Result of Simulation shows the impact of Gedunin on apoptosis regulation

The cumulative effect of selected neem compounds was more considerable on the regulation of apoptosis(lime green peak) and inhibition of inflammatory pathway(mint green color) by the rate of four as provided in figure 4.11.

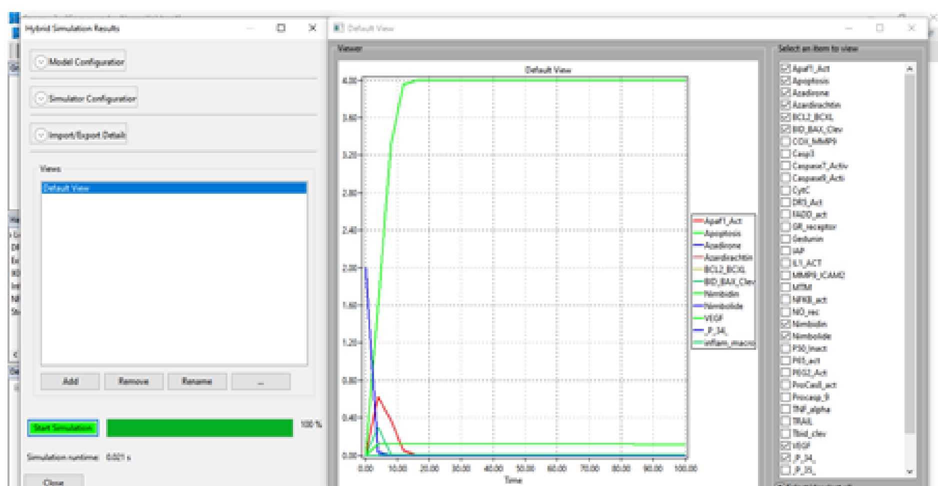

FIGURE 4.12: The effect of the *A. indica* compound on apoptosis and Inflammatory pathway. Table 4.2 provide detailed information on each entity after exposure to compounds

4.4 Lipinski's and ADMET properties of Phyto-compounds

In this study nimbidin and nimbolide act following all the Lipinski's property. The azadirone compound violates one property (log P value around 5). Similarly, azadiractin disrupts two properties H.B acceptance value greater than 10 and

molecular weight exceeds 500. In addition to this gedunin violate two rules; log P value 3.5 and molecular weight greater than 500.

A. Indica (neem) is the storehouse of various phytochemicals. The impact of 300 phyto-compounds natural products upon modern drug discovery compounds had been reported. There were a total of 1555 articles available for the given search term "Azadirachta indica [MeSH term]", 371 articles for "anti-cancer agent [drug]" and a total of 30 articles contain both terms in abstract or title. The five major compounds (azadirone, nimbolide, nimbidin, azadirachtin, gedunin) were selected from literature in the current study which were involved in the cancer by regulating inflammation and stimulating apoptosis.

In this study nimbidin and nimbolide act following all the Lipinski's property. The azadirone compound violates one property (log P value around 5). Similarly, azadiractin disrupts two properties H.B acceptance value greater than 10 and molecular weight exceeds 500. In addition to this gedunin violate two rules; log P value 3.5 and molecular weight greater than 500.

All the neem compounds have a high Gastrointestinal tract absorption value excluding azadirachtin due to high molecular weight. Similarly, all compounds are bio-available which was indicated by their given value around 0.5 which provided that compounds were suitable for oral uptake as they were readily available by oral ingestion. The azadirachtin doesn't have GI value. All the neem compounds have a high Gastrointestinal tract absorption value excluding azadirachtin due to high molecular weight. Similarly, all compounds are bio-available which was indicated by their given value around 0.5 which provided that compounds were suitable for oral uptake as they were readily available by oral ingestion. The azadirachtin doesn't have GI value that was due to violation of the Lipinski rule with the addition of rings in its structure. The value of CYP inhibitors was also good for all compounds except azadirone that violates 1. The CYP value indicated that compounds were transformed into other compounds in the body with the help of CYP enzymes that indicated that they are good drug targets. The detail of all the properties was provided in table 4.1 4.1.

Table 4.1: ADMET prediction of A.indica identified phyto-compounds

Parameters	**Azadirachtin (Meliacarpin)**	**Nimbolide**	**Azadirone**	**Gedunin**	**Nimbidin**
Pubchemid	183572	100017	10906239	12004512	25446
Physio-chemical properties					
Formula	$C_{27}H_{36}O_{11}$	$C_{27}H_{30}O_7$	$C_{28}H_{36}O_4$	$C_{28}H_{34}O_7$	$C_{26}H_{30}O_8$
Molecular weight	536.57 g/mol	466.52 g/mol	436.58 g/mol	482.57 g/mol	470.51 g/mol
Num. heavy atoms	38	34	32	35	34
Num. arom. heavy atoms	0	5	5	5	5
Fraction Csp3	0.89	0.59	0.64	.68	0.58
Num. rotatable bonds	3	4	3	3	4
Num. H-bond acceptors	11	7	4	7	8
Num. H-bond donors	4	0	0	0	3
Molar Refractivity	125.21	120.99	125.28	126.04	120.43
TPSA	156.67 $Å^2$	92.04 $Å^2$	-	95.34 $Å^2$	134.27 $Å^2$
Pharmaco-kinetics					
GI absorption	Low	High	High	High	High
CYP1A2 inhibitor	No	No	No	No	No

Parameters	**Azadirachtin (Meliacarpin)**	**Nimbolide**	**Azadirone**	**Gedunin**	**Nimbidin**
CYP2C19 inhibitor	No	No	No	No	No
CYP2C9 inhibitor	No	No	yes	No	No
CYP2D6 inhibitor	No	No	No	No	No
CYP3A4 inhibitor	No	No	No	No	No
Drug-likeness					
Lipinski	No; 2 violations: MW>500, NorO>10	Yes; 0 violation	Yes; 1 violation: MLOGP>4.15	No; 2 violations: MW>350, XLOGP3>3.5	Yes; 0 violation
Bioavailability Score	No; 3 violations: TPSA>150, #rings>7, H-acc>10	0.55	0.55	.55	0.56

4.5 Docking Studies

The inhibition potential of compounds/ligands and quality of ligand interaction was determined by the Root to mean square deviation (RMSD) value along with interacting amino acids. The value two are considered good and accepted overall. The value above two was considered as poor or unacceptable conformation. For every compound 2 interacting were considered for discussion having good RMSD value and significant residues involved in interaction [35].

The docking of Nimbidin with interacting proteins was provided in figure 4.13. This was the only compound that was involved in the inflammatory pathway of cancer. The two proteins interacted with compounds IL1B and PGE2. For PGE2 protein the residue involved in the binding pocket were (ARG#4, ASP#3, GLN#0, GLY#7, ILE#8, LYS#0, PHE#11, SER#0, TRP#4, TYR#0, ALA#3, VAL#7, ASN#5, CYS#2, GLU#0, HIS#2, LEU#10, MET#0, PRO#17, THR#8) with the value of drug score 0.78. The docking study provided two amino acids (Gly165) and (Lys160) form a hydrogen bond with the oxygen group of nimbin by acting as site chain donors. The RMSD value of nimbidin with PGE2 is 1.22. The RMSD value of ILIB with nimbidin was provided 1.22 and amino acids involved in the binding pocket were (ARG#0, ASP#0, GLN#0, GLY#0, ILE#2, LYS#,1 PHE#3, SER#0, TRP#0, TYR#,0 ALA#0, VAL#1, ASN#0, CYS#2, GLU#0, HIS#0, LEU#0, MET#0, PRO#0, THR#1). The docking study provided three amino acids involved in binding (Tyr24) form a hydrogen bond with OH group thus act as a backbone acceptor, the remaining two residues (Thr79, Gln81) form a hydrogen bond with OH group of Nimbin by acting as side chain acceptor.

The docking of azadirachtin with interacting proteins was provided in Table 4.2. The two proteins were selected for discussion based on the lowest RMSD value, Binding energy, and interacting residues P21 and PCNA. For P21 protein the residue involved in binding pocket were (ARG#1, ASP#0, GLN#0, GLY#0, ILE#1, LYS#,2 PHE#0, SER#0, TRP#0, TYR#0, ALA#0, VAL#2, ASN#0, CYS#1, GLU#0, HIS#0, LEU#0, MET#1, PRO#0, THR#0) with drug score 0.21. The simulation and docking provided 2 amino acids having 4 interactions: (Asn197) acted as side chain acceptor with OH group and site chain donor with

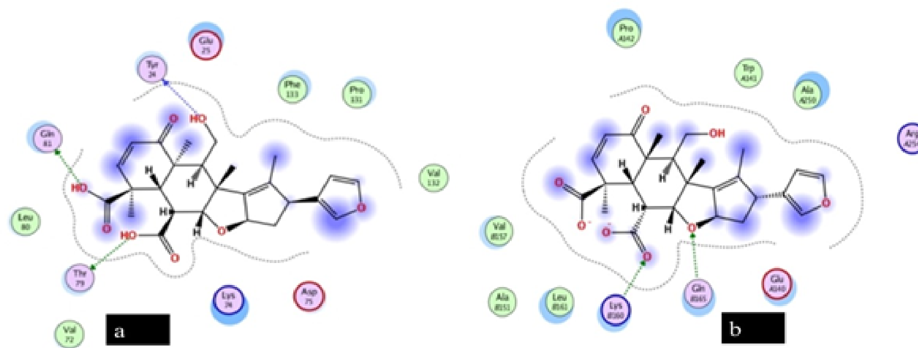

FIGURE 4.13: The binding of nimbolide: IL1B(a) involved three amino acids(Thr79), (Tyr24) and (Gln81); PEG2 two amino acids (GlnB165), (LysB260)

O group of azadirachtin compound through the formation of hydrogen bond, (Lys202) formed hydrogen bond by acting as site chain donor with OH and O group of azadirachtin with 0.75 RMSD value that was considered good. The interacting residue involved in predicting binding pockets of PCNA were (ARG#1, ASP#1, GLN#0, GLY#1, ILE#3, LYS#1, PHE#3, SER#1, TRP#1, TYR#0, ALA#4, VAL#2, ASN#1, CYS#0, GLU#1, HIS#0, LEU#8, MET#3, PRO#0, THR#2) with drug score 0.87. The four amino acids contributed to six interactions: (GluA85) acted as site chain acceptor with OH group, (Lys110) acted as backbone donor with OH group and site-chain donor with O group of azadirachtin, (GluB143) acted as site chin acceptor with H and OH group and (Arg146) acted as site chain acceptor with OH group with 1.08 RMSD value considered good for interaction. The docking result of both proteins was provided in (Figure 4.14).

TABLE 4.2: The RMSD value of interacting proteins with Azadirachtin

No.	Interacted Protein	RMSD Value
1	P21	0.75101566
2	PCNA	1.0861777
3	P53	1.1490865
4	BID	1.4708469
5	BAX	1.8423616
6	BCL2	2.044281
7	NCXL	2.1786957

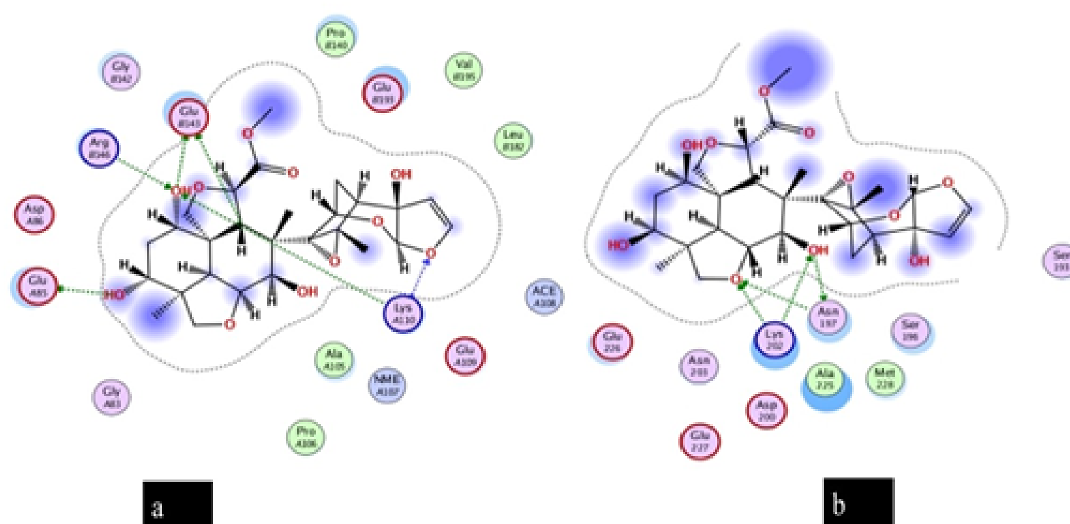

FIGURE 4.14: The binding of Azadirachtin: PCNA (a) involved four amino acids(GluA85), (LysA110), (GluA85) and (ArgB146); P21 two amino acids (Asn197), (Lys202)

The docking of nimbolide with interacting proteins was provided in Table 4.3. The two proteins were selected based on RMSD value, Binding energy, and interacting residues PTGS2 and VEGF. For PTGS2 protein the residue involved in the binding pocket were (ARG#5, ASP#1, GLN#5, GLY#4, ILE#2, LYS#4, PHE#3, SER#3, TRP#1, TYR#2, ALA#3, VAL#2, ASN#3, CYS#3, GLU#2, HIS#2, LEU#3, MET#1, PRO#9, THR#6) with drug score 0.94. The docking study provided three interacting amino acids (SerB126) and (HisB122) provided pi-pi interactions and (ArgB61) acted as site chain donor with oxygen group of ligand by forming a hydrogen bond. The overall result of docking was shown in figure 4.15. The interacting residue involved in predicting binding pockets of VEGF were (ARG#0, ASP#2, GLN#0, GLY#1, ILE#2, LYS#2, PHE#2, SER#2, TRP#0, TYR#3, ALA#0, VAL#1, ASN#1, CYS#4, GLU#3, HIS#0, LEU#2, MET#0, PRO#1, THR#0) with drug score 0.76. The docking simulation provided three interacting residues of amino acids (GlyB1) acted as backbone donor with oxygen group of compound,(MetB10) was involved in forming pi-pi aromatic interaction and (GluB14) acted as site chain acceptor by forming a hydrogen bond with C of nimbolide. The RMSD value that was 2 is considered good for interaction.

TABLE 4.3: The RMSD value of interacting proteins with Nimbolide

No.	Interacted Protein	RMSD Value
1	VEGF	1.427745
2	PTGS2	1.4925954
3	BCXL	1.701098
4	BIRC3	1.8434786
5	ICAM2	1.8761915
6	MMP9	2.0072684
7	BCL2	2.494452

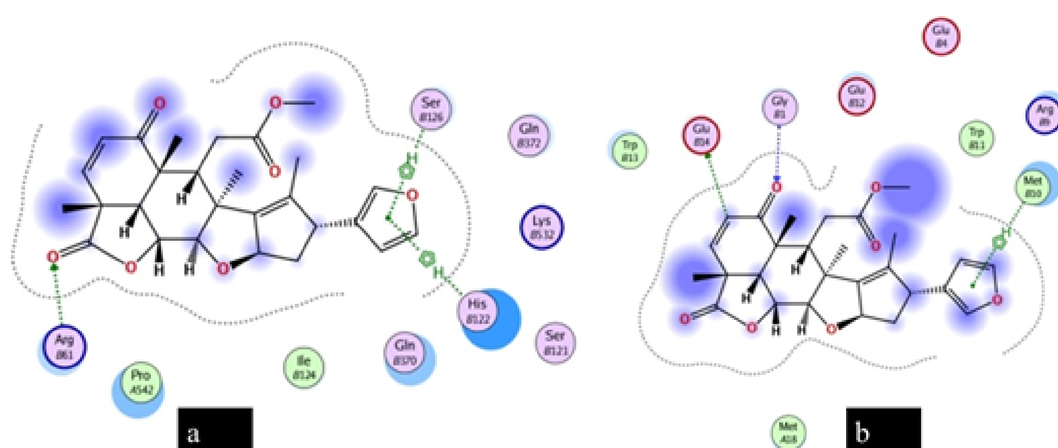

FIGURE 4.15: The binding of Nimbolide: PTGS2 (a) involved three amino acids with oxygen (ArgB61), (His122) and (SerB126); VEGF comprises amino acids (GluB14), (GlyB1), (Met B10)

Table 4.4 provided the docking result of interacting proteins with azadirone(100017). The Azardirone interacts with 7 proteins that were involved in initiating apoptosis. The best two were discussed based on RMSD value, drug score, and number of interacting residues in the binding pocket. The XIAP had residue involved in the binding pocket were (ARG#3, ASP#1, GLN#0, GLY#2, ILE#1, LYS#1, PHE#1, SER#2, TRP#0, TYR#1, ALA#0, VAL#0, ASN#0, CYS#0, GLU#1, HIS#0, LEU#3, MET#0, PRO#2, THR#0) 0.73 drug score. The XIAP had 4 binding sites with given compounds; (Arg20) with oxygen contributed to hydrogen bonding formation, (Asp11) act as site chain acceptor by contributing hydrogen

bond. The (Lys12) and (Leu27) form pi-pi aromatic interaction with benzene group. The RMSD value of this interaction is considered good 1.93 as provided in (figure 4.16). The SURVIVIN had residue involved in the binding pocket were (ARG#3, ASP#2, GLN#2, GLY#1, ILE#2, LYS#5, PHE#8, SER#1, TRP#1, TYR#0, ALA#4, VAL#1, ASN#0, CYS#2, GLU#2, HIS#0, LEU#6, MET#0, PRO#3, THR#1) and 0.81 drug score. The three interactions were provided by docking studies; the (Arg106) contribute to hydrogen bond formation with the oxygen of azadirone, (Phe93) and (Thr5) form hydrogen bond by acting as site chain acceptor with oxygen group. The overall RMSD value is considered as good for interaction that was (1.73).

TABLE 4.4: The RMSD value of Proteins with Azardirone

No.	Interacted Protein	RMSD Value
1	SURIVIN	1.7317441
2	XIAP	1.9391009
3	CFLAR	1.9696465
4	LRP8	2.1903079
5	BIRC2	2.469259
6	BCLXL	3.0998333
7	BCL2	7.3167543

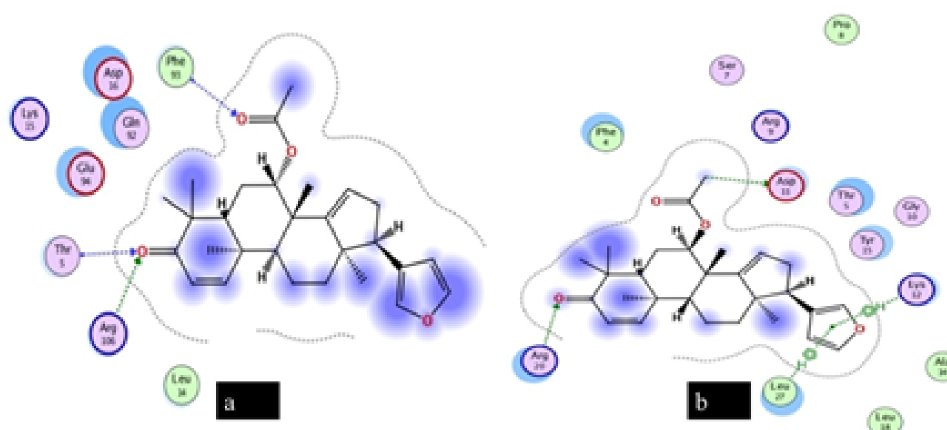

FIGURE 4.16: The binding of Azadirone: SURVIVIN (a) comprises three amino acids with oxygen (Phe93), (Thr5) and (Arg106); XIAP comprises four amino acids (Arg20), (Asp11), (Leu27) and (Lys12)

Chapter 5

Conclusion and Future Work

In this study, the stepwise modeling and analysis of *A. indica* compounds are revealed. Among 300 reported compounds five (Azadirone, Nimbolide, Nimbidin, Azadirachtin, Gedunin) are identified for modeling and analysis which are involved in an anti-cancer activity by modulating apoptosis and inflammatory pathways from text mining technique that achieved first objective.

The second objective was attained by modeling of normal and disease pathways revealed that certain factors trigger these processes. In the case of the extrinsic process of apoptosis, the binding of the ligand with death receptor-like FADD/-TRAIL is required for regulation. However, in the case of the intrinsic pathway, the certain checkpoint protein activates pro-apoptotic and inhibits anti-apoptotic proteins. The regulation of anti-apoptotic protein and pro-apoptotic protein along with death receptor modification leads to the immortality of cancerous cells. The inflammation involves the accumulation of cells that respond to tissue and infection damage. In cancer, the NFKB pathway that is triggered by ROS production and chemotactic attraction of leukocytes by IL1 production and PGE2 aggravate the process of inflammation.

The hybrid model provided the cumulative impact of *A. indica* compounds (by providing initial concentration) that control the rate of apoptosis and inflammatory pathways in cancer cells by triggering apoptosis and controlling the inflammatory response. The azardirachtin triggers apoptosis by upregulating pro-apoptotic proteins and downregulate cell survival proteins (pro-apoptotic proteins).

The azadirone targets overall six cell survival proteins(pro-apoptotic proteins) by inhibiting their expression and regulating apoptosis. However, nimbolide target cystine residue so that IKK proteins are unable to perform angiogenesis, growth, proliferation, and survival by targeting 7 proteins(VEGF, BCXL, BIRC3, ICAM2, MMP9, BCL2,PTGS2). Among five compounds nimbidin targets PEG2, IL1, and iNOS production that plays a significant role in controlling macrophage's inflammatory response. The cumulative impact of the compound on anti-cancer activity is quite significant by promoting apoptosis at the rate of four and inflammation at 0.2 that accomplished third objective.

The forth objective was fulfilled by drug likeliness property and docking study further provided that all compounds follow Lipinski's rule and are CYP inhibitors among them azardirone violates one rule and gedunin violate 2 rules. So, the rest of the compounds are involved in drug formation by obeying all the properties of ADMET.

The leading barrier in cancer treatment against individual pathway is the susceptibility of malignant tumor to adopt alternative pathways. However cumulative effect was studied only for two *A. indica* compounds nimbolide and azardirachtin that arrests multiple molecules by triggering mitrocondrial apoptosis offer greater potential as anticancer therapeutic drugs. Similarly this improve the therapeutic value of *A. indica* anti-cancerous compounds due to their carcinogenesis activity in vitro at minimal concentration.Hence improvement in multi-targated mechanism-based therapeutic strategies by plants natural products that display biological activity, affordability, drug target and lack of toxic effects would facilitate in improve drug design for cancer treatment.

Appendix A

Appendix

Table A.1: The effect of external and internal factors in regulation of normal process of apoptosis and inflammation

Time	Apoptosis	BCL2 BCXL	BID	Bax	COX MMP9	Caspase3 act
0	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
1	2.58E-07	2.68E-01	3.07E-01	1.91E-02	2.68E-01	2.77E-06
2	2.64E-04	5.13E-01	4.91E-01	1.50E-01	5.13E-01	1.19E-03
3	7.37E-03	6.57E-01	3.95E-01	2.94E-01	6.57E-01	1.80E-02
4	4.91E-02	7.09E-01	2.61E-01	3.44E-01	7.09E-01	7.19E-02
5	1.59E-01	7.22E-01	1.63E-01	3.12E-01	7.22E-01	1.50E-01
6	3.45E-01	7.25E-01	1.01E-01	2.44E-01	7.25E-01	2.16E-01
7	5.81E-01	7.25E-01	6.41E-02	1.75E-01	7.25E-01	2.49E-01
8	8.32E-01	7.25E-01	4.24E-02	1.20E-01	7.25E-01	2.48E-01
9	1.07E+00	7.25E-01	2.94E-02	8.09E-02	7.25E-01	2.22E-01
10	1.27E+00	7.25E-01	2.13E-02	5.45E-02	7.25E-01	1.85E-01
11	1.44E+00	7.25E-01	1.61E-02	3.73E-02	7.25E-01	1.47E-01
12	1.57E+00	7.25E-01	1.26E-02	2.63E-02	7.25E-01	1.12E-01
13	1.66E+00	7.25E-01	1.01E-02	1.92E-02	7.25E-01	8.33E-02

Time	Apoptosis	BCL2 BCXL	BID	Bax	COX MMP9	Caspase3 act
14	1.74E+00	7.25E-01	8.29E-03	1.45E-02	7.25E-01	6.11E-02
15	1.79E+00	7.25E-01	6.93E-03	1.13E-02	7.25E-01	4.45E-02
16	1.83E+00	7.25E-01	5.89E-03	9.04E-03	7.25E-01	3.25E-02
17	1.85E+00	7.25E-01	5.07E-03	7.43E-03	7.25E-01	2.39E-02
18	1.88E+00	7.25E-01	4.41E-03	6.23E-03	7.25E-01	1.79E-02
19	1.89E+00	7.25E-01	3.87E-03	5.30E-03	7.25E-01	1.36E-02
20	1.90E+00	7.25E-01	3.43E-03	4.58E-03	7.25E-01	1.07E-02

Table A.2: The effect of external and internal factors in regulation of normal process of apoptosis and inflammation

Time	Caspase9 act	Checkpoint Protein	CytoC ACT	Extra Cellular Ligand	ForeginParticles Virus Bacteria
0	0.00E+00	2.00E+00	0.00E+00	2.00E+00	2.00E+00
1	6.91E-05	7.36E-01	4.98E-04	7.36E-01	7.36E-01
2	4.62E-03	2.71E-01	1.76E-02	2.71E-01	2.71E-01
3	2.69E-02	9.96E-02	8.32E-02	9.96E-02	9.96E-02
4	6.20E-02	3.66E-02	1.79E-01	3.66E-02	3.66E-02
5	9.47E-02	1.35E-02	2.55E-01	1.35E-02	1.35E-02
6	1.17E-01	4.96E-03	2.83E-01	4.96E-03	4.96E-03
7	1.26E-01	1.82E-03	2.67E-01	1.82E-03	1.82E-03
8	1.23E-01	6.71E-04	2.24E-01	6.71E-04	6.71E-04
9	1.11E-01	2.47E-04	1.75E-01	2.47E-04	2.47E-04
10	9.52E-02	9.08E-05	1.29E-01	9.08E-05	9.08E-05
11	7.74E-02	3.34E-05	9.18E-02	3.34E-05	3.34E-05

Time	Caspase9 act	Checkpoint Protein	CytoC ACT	Extra Cellular Ligand	ForeginParticles Virus Bacteria
12	6.07E-02	1.23E-05	6.44E-02	1.23E-05	1.23E-05
13	4.63E-02	4.52E-06	4.50E-02	4.52E-06	4.52E-06
14	3.48E-02	1.66E-06	3.18E-02	1.66E-06	1.66E-06
15	2.60E-02	6.12E-07	2.29E-02	6.12E-07	6.12E-07
16	1.96E-02	2.25E-07	1.69E-02	2.25E-07	2.25E-07
17	1.49E-02	8.28E-08	1.28E-02	8.28E-08	8.28E-08
18	1.16E-02	3.05E-08	1.01E-02	3.05E-08	3.05E-08
19	9.23E-03	1.12E-08	8.09E-03	1.12E-08	1.12E-08
20	7.49E-03	4.12E-09	6.66E-03	4.12E-09	4.12E-09

Table A.3: The effect of external and internal factors in regulation of normal process of apoptosis and inflammation

Time	IAP	IL1 ACT	Mitrocondria Trans Membrane Apaf1	NO enh	P50 act
0	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.00E+00
1	2.68E-01	4.36E-01	3.35E-03	9.64E-01	1.37E+00
2	5.13E-01	2.44E-01	5.63E-02	1.43E+00	1.14E+00
3	6.57E-01	8.79E-02	1.72E-01	1.69E+00	1.05E+00
4	7.09E-01	2.75E-02	2.73E-01	1.84E+00	1.02E+00
5	7.22E-01	8.58E-03	3.09E-01	1.93E+00	1.01E+00
6	7.25E-01	2.81E-03	2.86E-01	1.97E+00	1.00E+00
7	7.25E-01	9.70E-04	2.34E-01	1.99E+00	1.00E+00
8	7.25E-01	3.45E-04	1.76E-01	1.99E+00	1.00E+00

Time	IAP	IL1 ACT	Mitrocondria Trans Membrane Apaf1	NO enh	P50 act
9	7.25E-01	1.25E-04	1.25E-01	2.00E+00	1.00E+00
10	7.25E-01	4.57E-05	8.70E-02	2.00E+00	1.00E+00
11	7.25E-01	1.67E-05	5.98E-02	2.00E+00	1.00E+00
12	7.25E-01	6.15E-06	4.13E-02	2.00E+00	1.00E+00
13	7.25E-01	2.26E-06	2.90E-02	2.00E+00	1.00E+00
14	7.25E-01	8.32E-07	2.10E-02	2.00E+00	1.00E+00
15	7.25E-01	3.06E-07	1.56E-02	2.00E+00	1.00E+00
16	7.25E-01	1.13E-07	1.20E-02	2.00E+00	1.00E+00
17	7.25E-01	4.14E-08	9.51E-03	2.00E+00	1.00E+00
18	7.25E-01	1.52E-08	7.74E-03	2.00E+00	1.00E+00
19	7.25E-01	5.60E-09	6.43E-03	2.00E+00	1.00E+00
20	7.25E-01	2.06E-09	5.44E-03	2.00E+00	1.00E+00

TABLE A.4: The effect of external and internal factors in regulation of normal process of apoptosis and inflammation

Time	P65 act	PEG2 Act	PUMA Act	ProCaspase8 act	TNF alpha
0	0.00E+00	1.00E+00	2.00E+00	0.00E+00	1.00E+00
1	2.89E-01	3.68E-01	7.36E-01	4.25E-01	3.68E-01
2	2.34E-01	1.35E-01	2.71E-01	1.24E+00	1.45E-01
3	2.12E-01	4.98E-02	9.96E-02	1.93E+00	1.07E-01
4	2.05E-01	1.83E-02	3.66E-02	2.31E+00	1.41E-01
5	2.02E-01	6.74E-03	1.35E-02	2.40E+00	1.73E-01
6	2.01E-01	2.48E-03	4.96E-03	2.30E+00	1.89E-01

Time	P65 act	PEG2 Act	PUMA Act	ProCaspase8 act	TNF alpha
7	2.00E-01	9.12E-04	1.82E-03	2.10E+00	1.96E-01
8	2.00E-01	3.35E-04	6.71E-04	1.89E+00	1.99E-01
9	2.00E-01	1.23E-04	2.47E-04	1.70E+00	2.00E-01
10	2.00E-01	4.54E-05	9.08E-05	1.54E+00	2.00E-01
11	2.00E-01	1.67E-05	3.34E-05	1.41E+00	2.00E-01
12	2.00E-01	6.14E-06	1.23E-05	1.32E+00	2.00E-01
13	2.00E-01	2.26E-06	4.52E-06	1.25E+00	2.00E-01
14	2.00E-01	8.31E-07	1.66E-06	1.20E+00	2.00E-01
15	2.00E-01	3.06E-07	6.12E-07	1.17E+00	2.00E-01
16	2.00E-01	1.13E-07	2.25E-07	1.14E+00	2.00E-01
17	2.00E-01	4.14E-08	8.28E-08	1.12E+00	2.00E-01
18	2.00E-01	1.52E-08	3.05E-08	1.11E+00	2.00E-01
19	2.00E-01	5.60E-09	1.12E-08	1.10E+00	2.00E-01
20	2.00E-01	2.06E-09	4.12E-09	1.09E+00	2.00E-01

Table A.5: The effect of external and internal factors in regulation of normal process of apoptosis and inflammation

Time	Tbid cleav	VEGF	P32	iNOS incre	inflam macro
0	0.00E+00	0.00E+00	0.00E+00	1.00E+00	0.00E+00
1	8.78E-02	2.68E-01	0.00E+00	4.08E-01	7.89E-01
2	3.17E-01	5.13E-01	0.00E+00	3.01E-01	1.32E+00
3	3.97E-01	6.57E-01	0.00E+00	1.78E-01	1.68E+00
4	3.45E-01	7.09E-01	0.00E+00	7.90E-02	1.88E+00
5	2.54E-01	7.22E-01	0.00E+00	2.83E-02	1.96E+00
6	1.72E-01	7.25E-01	0.00E+00	8.71E-03	1.99E+00

Time	Tbid cleav	VEGF	P32	iNOS incre	inflam macro
7	1.12E-01	7.25E-01	0.00E+00	2.47E-03	2.00E+00
8	7.33E-02	7.25E-01	0.00E+00	6.93E-04	2.00E+00
9	4.87E-02	7.25E-01	0.00E+00	2.01E-04	2.00E+00
10	3.33E-02	7.25E-01	0.00E+00	6.14E-05	2.00E+00
11	2.37E-02	7.25E-01	0.00E+00	1.95E-05	2.00E+00
12	1.75E-02	7.25E-01	0.00E+00	6.35E-06	2.00E+00
13	1.34E-02	7.25E-01	0.00E+00	2.10E-06	2.00E+00
14	1.06E-02	7.25E-01	0.00E+00	7.04E-07	2.00E+00
15	8.63E-03	7.25E-01	0.00E+00	2.38E-07	2.00E+00
16	7.16E-03	7.25E-01	0.00E+00	8.11E-08	2.00E+00
17	6.05E-03	7.25E-01	0.00E+00	2.78E-08	2.00E+00
18	5.18E-03	7.25E-01	0.00E+00	9.56E-09	2.00E+00
19	4.49E-03	7.25E-01	0.00E+00	3.30E-09	2.00E+00
20	3.93E-03	7.25E-01	0.00E+00	1.15E-09	2.00E+00

Table A.6: Impact of anti-apoptotic protein on apoptosis and iNOS on inflammatory pathways

Time	Apoptosis	BCL2 BCXL	BH3 Domain	BID	COX MMP9	Caspase3 act
0	0	0	0	0	0	0
5	0	1.97E-01	1.99E+00	6.93E-01	1.97E-01	0
10	0	1.98E-01	2.00E+00	6.93E-01	1.98E-01	0
15	0	1.96E-01	2.00E+00	6.93E-01	1.96E-01	0
20	0	1.95E-01	2.00E+00	6.93E-01	1.95E-01	0
25	0	1.94E-01	2	6.93E-01	1.94E-01	0
30	0	1.92E-01	2	6.93E-01	1.92E-01	0

Time	Apoptosis	BCL2 BCXL	BH3 Domain	BID	COX MMP9	Caspase3 act
35	0	1.91E-01	2	6.93E-01	1.91E-01	0
40	0	1.90E-01	2	6.93E-01	1.90E-01	0
45	0	1.89E-01	2	6.93E-01	1.89E-01	0
50	0	1.87E-01	2	6.93E-01	1.87E-01	0
55	0	1.86E-01	2	6.93E-01	1.86E-01	0
60	0	1.85E-01	2	6.93E-01	1.85E-01	0
65	0	1.84E-01	2	6.93E-01	1.84E-01	0
70	0	1.83E-01	2	6.93E-01	1.83E-01	0
75	0	1.82E-01	2	6.93E-01	1.82E-01	0
80	0	1.81E-01	2	6.93E-01	1.81E-01	0
85	0	1.80E-01	2	6.93E-01	1.80E-01	0
90	0	1.79E-01	2	6.93E-01	1.79E-01	0
95	0	1.78E-01	2	6.93E-01	1.78E-01	0
100	0	1.77E-01	2	6.93E-01	1.77E-01	0

Table A.7: Impact of anti-apoptotic protein on apoptosis and iNOS on inflammatory pathways

Time	Caspase9 act	Checkpoint Protein	CytoC ACT	DR5 Inact	Foregin Particles Virus Bacteria
0	0	2	0	0	2
5	4.01E+00	1.35E-02	9.93E-01	5	1.35E-02
10	8.32E+00	9.08E-05	2.37E-01	10	9.08E-05
15	8.56E+00	6.12E-07	1.59E-03	15	6.12E-07
20	8.56E+00	4.12E-09	1.07E-05	20	4.12E-09
25	8.56E+00	2.78E-11	7.24E-08	25	2.78E-11

Time	Caspase9 act	Checkpoint Protein	CytoC ACT	DR5 Inact	Foreign Particles Virus Bacteria
30	8.56E+00	1.87E-13	4.88E-10	30	1.87E-13
35	8.56E+00	1.26E-15	3.29E-12	35	1.26E-15
40	8.56E+00	8.50E-18	2.21E-14	40	8.50E-18
45	8.56E+00	5.72E-20	1.49E-16	45	5.72E-20
50	8.56E+00	3.86E-22	1.01E-18	50	3.86E-22
55	8.56E+00	2.60E-24	6.77E-21	55	2.60E-24
60	8.56E+00	1.75E-26	4.56E-23	60	1.75E-26
65	8.56E+00	1.18E-28	3.08E-25	65	1.18E-28
70	8.56E+00	7.95E-31	2.07E-27	70	7.95E-31
75	8.56E+00	5.36E-33	1.40E-29	75	5.36E-33
80	8.56E+00	3.61E-35	9.41E-32	80	3.61E-35
85	8.56E+00	2.43E-37	6.34E-34	85	2.43E-37
90	8.56E+00	1.64E-39	4.27E-36	90	1.64E-39
95	8.56E+00	1.10E-41	2.88E-38	95	1.10E-41
100	8.56E+00	7.44E-44	1.94E-40	100	7.44E-44

TABLE A.8: Impact of anti-apoptotic protein on apoptosis and iNOS on inflammatory pathways

Time	IAP	IL1 ACT	MDM2 inhibition	MMP9 ICAM2	Mitrocondria Trans Membrane Apaf1
0	0	0	0	0	0
5	1.97E-01	8.58E-03	1.99E+00	1.97E-01	9.89E-01
10	1.98E-01	4.57E-05	2.00E+00	1.98E-01	1.00E+00

Time	IAP	IL1 ACT	MDM2 inhibition	MMP9 ICAM2	Mitrocondria Trans Membrane Apaf1
15	1.96E-01	3.06E-07	2.00E+00	1.96E-01	1.00E+00
20	1.95E-01	2.06E-09	2.00E+00	1.95E-01	1.00E+00
25	1.94E-01	1.39E-11	2	1.94E-01	1.00E+00
30	1.92E-01	9.36E-14	2	1.92E-01	1.00E+00
35	1.91E-01	6.30E-16	2	1.91E-01	1.00E+00
40	1.90E-01	4.25E-18	2	1.90E-01	1.00E+00
45	1.89E-01	2.86E-20	2	1.89E-01	1.00E+00
50	1.87E-01	1.93E-22	2	1.87E-01	1.00E+00
55	1.86E-01	1.30E-24	2	1.86E-01	1.00E+00
60	1.85E-01	8.76E-27	2	1.85E-01	1.00E+00
65	1.84E-01	5.90E-29	2	1.84E-01	1.00E+00
70	1.83E-01	3.98E-31	2	1.83E-01	1.00E+00
75	1.82E-01	2.68E-33	2	1.82E-01	1.00E+00
80	1.81E-01	1.80E-35	2	1.81E-01	1.00E+00
85	1.80E-01	1.22E-37	2	1.80E-01	1.00E+00
90	1.79E-01	8.19E-40	2	1.79E-01	1.00E+00
95	1.78E-01	5.52E-42	2	1.78E-01	1.00E+00
100	1.77E-01	3.72E-44	2	1.77E-01	1.00E+00

Table A.9: Impact of anti-apoptotic protein on apoptosis and iNOS on inflammatory pathways

Time	NFKB act	NO enh	O2 Release	P50 act	P65 act	PEG2 Act
0	0	0	1	0	0	1
5	6.12E-01	1.93E+00	7.41E-02	1.16E-02	2.90E-03	6.74E-03

Time	NFKB act	NO enh	O2 Release	P50 act	P65 act	PEG2 Act
10	6.04E-01	2.00E+00	9.53E-04	7.80E-05	1.95E-05	4.54E-05
15	6.06E-01	2.00E+00	9.48E-06	5.26E-07	1.32E-07	3.06E-07
20	6.07E-01	2.00E+00	8.45E-08	3.54E-09	1.03E-09	2.06E-09
25	6.09E-01	2.00E+00	7.08E-10	2.39E-11	6.79E-10	1.39E-11
30	6.10E-01	2	5.71E-12	1.61E-13	0	9.36E-14
35	6.11E-01	2	4.48E-14	1.08E-15	8.60E-12	6.30E-16
40	6.13E-01	2	3.44E-16	7.30E-18	1.76E-10	4.25E-18
45	6.14E-01	2	2.60E-18	4.92E-20	0	2.86E-20
50	6.15E-01	2	1.95E-20	3.32E-22	0	1.93E-22
55	6.16E-01	2	1.44E-22	2.23E-24	0	1.30E-24
60	6.17E-01	2	1.06E-24	1.51E-26	6.48E-10	8.76E-27
65	6.18E-01	2	7.73E-27	1.01E-28	4.15E-11	5.90E-29
70	6.19E-01	2	5.61E-29	6.83E-31	6.40E-12	3.98E-31
75	6.20E-01	2	4.04E-31	4.60E-33	2.24E-10	2.68E-33
80	6.21E-01	2	2.91E-33	3.10E-35	0	1.80E-35
85	6.22E-01	2	2.08E-35	2.09E-37	0	1.22E-37
90	6.23E-01	2	1.48E-37	1.41E-39	0	8.19E-40
95	6.24E-01	2	1.05E-39	9.49E-42	2.80E-10	5.52E-42
100	6.25E-01	2	7.48E-42	6.40E-44	7.79E-11	3.72E-44

Table A.10: Impact of anti-apoptotic protein on apoptosis and iNOS on inflammatory pathways

Time	PUMA Act	TNF alpha	VEGF	Bax	iNOS incre	inflam macro
0	2	1	0	0	0	0
5	1.35E-02	6.98E-03	1.97E-01	1.16E-02	2.64E-02	9.58E-01
10	9.08E-05	3.50E-04	1.98E-01	7.80E-05	1.55E-04	1.00E+00

Time	PUMA Act	TNF alpha	VEGF	Bax	iNOS incre	inflam macro
15	6.12E-07	2.95E-04	1.96E-01	5.26E-07	1.01E-06	1.00E+00
20	4.12E-09	2.84E-04	1.95E-01	3.54E-09	6.79E-09	1.00E+00
25	2.78E-11	2.74E-04	1.94E-01	2.39E-11	4.57E-11	1
30	1.87E-13	2.64E-04	1.92E-01	1.61E-13	3.07E-13	1
35	1.26E-15	2.56E-04	1.91E-01	1.08E-15	2.06E-15	1
40	8.50E-18	2.47E-04	1.90E-01	7.30E-18	1.39E-17	1
45	5.72E-20	2.40E-04	1.89E-01	4.92E-20	9.33E-20	1
50	3.86E-22	2.32E-04	1.87E-01	3.32E-22	6.28E-22	1
55	2.60E-24	2.25E-04	1.86E-01	2.23E-24	4.22E-24	1
60	1.75E-26	2.19E-04	1.85E-01	1.51E-26	2.84E-26	1
65	1.18E-28	2.12E-04	1.84E-01	1.01E-28	1.91E-28	1
70	7.95E-31	2.06E-04	1.83E-01	6.83E-31	1.28E-30	1
75	5.36E-33	2.01E-04	1.82E-01	4.60E-33	8.64E-33	1
80	3.61E-35	1.95E-04	1.81E-01	3.10E-35	5.81E-35	1
85	2.43E-37	1.90E-04	1.80E-01	2.09E-37	3.91E-37	1
90	1.64E-39	1.85E-04	1.79E-01	1.41E-39	2.63E-39	1
95	1.10E-41	1.81E-04	1.78E-01	9.49E-42	1.77E-41	1
100	7.44E-44	1.76E-04	1.77E-01	6.40E-44	1.19E-43	1

TABLE A.11: The cumulative effect of *A.indica* compounds on apoptosis and inflammatory pathways

Time	Apoptosis	Azadirone	Azardirachtin	Nimbidin	DR5 Act	MTM
0	0.00E+00	2.00E+00	2.00E+00	2.00E+00	0.00E+00	0.00E+00
5	4.72E-08	7.36E-01	7.36E-01	7.36E-01	3.68E-01	2.45E-01
10	1.95E-04	2.71E-01	2.71E-01	2.71E-01	5.41E-01	7.22E-01

Time	Apoptosis	Azadirone	Azardirachtin	Nimbidin	DR5 Act	MTM
15	1.05E-02	9.96E-02	9.96E-02	9.96E-02	4.48E-01	8.96E-01
20	9.38E-02	3.66E-02	3.66E-02	3.66E-02	2.93E-01	7.81E-01
25	3.27E-01	1.35E-02	1.35E-02	1.35E-02	1.68E-01	5.61E-01
30	6.75E-01	4.96E-03	4.96E-03	4.96E-03	8.92E-02	3.57E-01
35	1.03E+00	1.82E-03	1.82E-03	1.82E-03	4.47E-02	2.09E-01
40	1.32E+00	6.71E-04	6.71E-04	6.71E-04	2.15E-02	1.15E-01
45	1.52E+00	2.47E-04	2.47E-04	2.47E-04	1.00E-02	6.00E-02
50	1.65E+00	9.08E-05	9.08E-05	9.08E-05	4.54E-03	3.03E-02
55	1.73E+00	3.34E-05	3.34E-05	3.34E-05	2.02E-03	1.48E-02
60	1.79E+00	1.23E-05	1.23E-05	1.23E-05	8.85E-04	7.08E-03
65	1.82E+00	4.52E-06	4.52E-06	4.52E-06	3.82E-04	3.31E-03
70	1.85E+00	1.66E-06	1.66E-06	1.66E-06	1.63E-04	1.52E-03
75	1.87E+00	6.12E-07	6.12E-07	6.12E-07	6.88E-05	6.88E-04
80	1.88E+00	2.25E-07	2.25E-07	2.25E-07	2.88E-05	3.07E-04
85	1.90E+00	8.28E-08	8.28E-08	8.28E-08	1.20E-05	1.36E-04
90	1.91E+00	3.05E-08	3.05E-08	3.05E-08	4.93E-06	5.92E-05
95	1.91E+00	1.12E-08	1.12E-08	1.12E-08	2.02E-06	2.56E-05
100	1.92E+00	4.12E-09	4.12E-09	4.12E-09	8.24E-07	1.10E-05

Table A.12: The cumulative effect of *A.indica* compounds on apoptosis and inflammatory pathways

Time	Gedunin	BID BAX Clev	TRAIL	Nimbolide	Apaf1 Act	inflam macro
0	2.00E+00	0.00E+00	0.00E+00	2.00E+00	0.00E+00	0.00E+00
5	7.36E-01	1.47E+00	7.36E-01	7.36E-01	1.23E-02	3.75E-01

Time	Gedunin	BID BAX Clev	TRAIL	Nimbolide	Apaf1 Act	inflam macro
10	2.71E-01	1.08E+00	5.41E-01	2.71E-01	1.44E-01	3.75E-01
15	9.96E-02	5.97E-01	2.99E-01	9.96E-02	4.03E-01	3.75E-01
20	3.66E-02	2.93E-01	1.47E-01	3.66E-02	6.25E-01	3.75E-01
25	1.35E-02	1.35E-01	6.74E-02	1.35E-02	7.02E-01	3.75E-01
30	4.96E-03	5.95E-02	2.97E-02	4.96E-03	6.42E-01	3.75E-01
35	1.82E-03	2.55E-02	1.28E-02	1.82E-03	5.11E-01	3.75E-01
40	6.71E-04	1.07E-02	5.37E-03	6.71E-04	3.66E-01	3.75E-01
45	2.47E-04	4.44E-03	2.22E-03	2.47E-04	2.43E-01	3.75E-01
50	9.08E-05	1.82E-03	9.08E-04	9.08E-05	1.51E-01	3.75E-01
55	3.34E-05	7.35E-04	3.67E-04	3.34E-05	8.97E-02	3.75E-01
60	1.23E-05	2.95E-04	1.47E-04	1.23E-05	5.10E-02	3.75E-01
65	4.52E-06	1.18E-04	5.88E-05	4.52E-06	2.80E-02	3.75E-01
70	1.66E-06	4.66E-05	2.33E-05	1.66E-06	1.49E-02	3.75E-01
75	6.12E-07	1.84E-05	9.18E-06	6.12E-07	7.74E-03	3.75E-01
80	2.25E-07	7.20E-06	3.60E-06	2.25E-07	3.93E-03	3.75E-01
85	8.28E-08	2.82E-06	1.41E-06	8.28E-08	1.96E-03	3.75E-01
90	3.05E-08	1.10E-06	5.48E-07	3.05E-08	9.59E-04	3.75E-01
95	1.12E-08	4.26E-07	2.13E-07	1.12E-08	4.62E-04	3.75E-01
100	4.12E-09	1.65E-07	8.24E-08	4.12E-09	2.20E-04	3.75E-01

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