Chemoprofiling of Medicinal Plants and Value Addition of Their Principle Constituents through Synthetic Modifications

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BY

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Dedicated to my beloved parents for their love and affection….

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Certificate

This is to certify that the work presented in this dissertation entitled "***Chemoprofiling of medicinal plants and value addition of their principle constituents through synthetic modifications***" is original and has been carried out by **Mr Showkat Rashid** under our joint supervision. This work is suitable for submission for the award of M.Phil Degree in Chemistry. It is further certified that the work has not been submitted in part or full for award of any degree in this or any other University.

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Declaration

I hereby declare that the dissertation entitled **"*Chemoprofiling of medicinal plants and value addition of their principle constituents through synthetic modifications*"** submitted for M.Phil degree to the University of Kashmir has been carried out at Indian Institute of Integrative Medicine-Srinagar and department of chemistry, University of Kashmir; under the joint supervision of Dr Syed Wajaht Amin Shah (Department of Chemistry, University of Kashmir) and Dr Bilal Ahmad Bhat (Scientist, IIIM-Srinagar). The work embodied in this dissertation is original and has not been submitted in part or full for any degree or diploma to this or any other University.

Dated: **Showkat Rashid**

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Abstract

Natural products, the greatest treasures of nature, are continous and unending source of inspiration for mankind to haunt potentialy lead molecules for pharmaceutical industry. The search for these natural products in medicine dates back to 1550 BC, but the scientific period of this search is much more recent. Historically, plants were a folkloric source of medicinal agents and as modern medicine developed, numerous useful drugs were developed from lead compounds discovered from medicinal plants. Today, this strategy remains an essential route to new pharmaceuticals with multifaceted biological potential. The present investigation was undertaken to study, in particular, the bioprospection of historically relevant medicinal plant, *Cotula anthemoides*, and the value addition of its principal bioactive constituent, ursolic acid (UA), through rational structural modifications as potential anti-cancer agents.

Cotula, the largest genus of flowering plants in Southern Hemisphere which belongs to family *Asteraceae* and tribe Anthemideae. It constitutes roughly about 80 species of plants generally known as "water buttons" or "button weeds". *Cotula anthemoides* is a well-known member of this genus commonly known as '*Babuna*' and locally as "*Thulla bobul*". In traditional medicine, its roots were taken in Lesotho for colic and in South Africa it is a remedy for head and chest colds. Its extensive use in the world wide flu-epidemic of 1919 makes it an attactive target for future research. *Cotula anthemoides* plant material was collected from Kelar region of District Pulwama and its identification was assured in the Department of Plant Taxonomy, University of Kashmir. A specimen bearing Voucher Specimen No. KASH-710 was submitted to the same department. The aerial part of plant was dried, crushed and subjected to extraction by various organic solvents. Bioactivity guided extraction directed us to focus on methanolic extract. Owing to its potential cytotoxic and antimicrobial activity, methanolic extract was further subjected to column chromatography which resulted into the isolation of 11 individual constituents. Incisive spectral analysis enabled us to identify unambigously four known constituents (α-pinene, coumarin, ursolic acid, gibberlic acid A-3) and a new coumarin ester, 6-Methoxy-2-oxo-2H-chromene-8-carboxylic acid methylester all reported for the first time from this plant. All the isolated constituents were reevaluated against a panel of human cancer cell lines for anticancer activity and against bacterial and fungal strains for anti-microbial activity. It was interesting to note that, among the five compounds screened, ursolic acid and 6-Methoxy-2-oxo-2H-chromene-8-carboxylic acid methylester exhibited

potential anti-cancer activity against four cancer cell lines, THP-1 (leukaemia), A-549 (lung), PC-3 (Prostate) and HCT-15 (colon) and significant antifungal activity against two fungal strains, *Aspergillus niger* and *Penicillium chrysogenum.*

Taking cue from the preceeding discussion and as part of our ongoing research program to synthsesize biocative molecules as anti-cancer agents, we aim to undertake a research program to design and structuraly modify the UA in order to fine tune its anti-cancer potential through click chemistry approach. Accordingly, a series of UA- triazolyl derivatives were designed and synthesized by employing Cu (I) catalyzed 1, 3-dipolar cycloaddition reaction of propargylated-UA derivative with various aromatic azides. All the compounds were confimed by ^{1}HNMR, ^{13}C NMR, IR and ESI-MS analysis. In ^{1}HNMR, cyclization of azides to form triazoles, was confirmed by resonance of H-5 of triazole ring in aromatic region as well as by the presence of other protons in aromatic region. The structure was further supported by the ^{13}CNMR and DEPT, which showed all the expected carbon signals corresponding to triazole derivatives. All the UA-triazolyl derivatives were assayed for *in vitro* cytotoxicity against a panel of four human cancer cell lines including A-549 (lung), MCF-7 (breast), HCT-116 (colon), THP-1 (leukemia) and a normal human epithelial cell line, FR-2 using sulforhodamine-B assay. 5-fluorouracil and mitomycin-C in addition to UA were taken as reference standards. From the anticancer screening data, it was interesting to note that some of the compounds exhibited interresting anti-cancer activity. Compound **9c** which contains the *p*-bromo substitution at aryl ring was found to be most promising compound with a 2-25 fold decrease in IC_{50} value. All the UA-triazolyl derivatives were also screened for antimicrobial activity against seven bacteria and two fungal strains. The results indicate that these compounds displayed a broad spectrum and variable degree of antibacterial and antifungal activity against the different tested strains. Compounds **9d**, **9j** and **9k** were most promising antibacterial agents and compound **9b** and **9i** were found to be most promising antifungal compounds in this study.

Chapter -1

Introduction and review of literature

Introduction:

1.1. Historical persepective of medicinal plants:

Mankind has remained profoundly dependent on surrounding flora and fauna throughout its organic evolution. Superior intellect and extraordinary physical manoeuvrability made him to strive from the beginning to exploit all available resources to their advantage. Plants, mostly angiosperms, which are seed bearers readily invaded the dry land where man lived. This have provided him all the basic amenities for the maintenance of his livelihood. Plants directly provide man food-stuffs, fibres, shelter, raw materials for industry, like wood, waxes, rubbers, metals etc. These also provide ameliorants like essential oils, poisons, narcotics and above all the special substances for the upkeep of his health i.e., medicines. To trace the history of phytotherapy is to trace the history of humanity itself. The discovery of the preventive, promotive and curative properties of certain plants must have sprung from instinct. Primitive people first used plants as food and as a result of this desire; the link with some plant properties would have been learnt. Medicinal plants were the main source of products used to sustain health until the nineteenth century. Medicinal preparations derived from natural sources, especially from plants have been in widespread use since time immemorial. Ancient texts of India and China contain exhaustive depictions of the use of a variety of plant-derived medications. In fact, plants remain the main source of medicines for a large proportion of the world's population, particularly in the developing world, despite the advent of the pharmaceutical chemistry during the early twentieth century, which brought/coupled with it the ability to synthesize an enormous variety of medicinal drug molecules allowed mankind treatment of previously incurable life-threatening diseases.

Early in his history, man learnt that some plants have unusual effects on mind and body in addition to relieving aches and pains. He considered these plants as sacred and associated them with magic and religion. In the course of time, he learnt the therapeutic potential which ultimately culminated into "traditional or alternative source of medicine" and these plants which possessed therapeutic or prophylactic properties.

now what we call "the medicinal plants". World Health Organization (WHO) has formulated the definition for medicinal plants as those plants that possess therapeutic properties or exert beneficial pharmacological effects on animal / human body.

The application of plants as medicine dates back to prehistoric period. The early civilization reveals that a considerable number of drugs used in modern medicine have figured in ancient manuscripts such as the Rigved, The Bible, The Holy Quran and the History of Herodotus. Over 6000 years ago, the ancient Chinese were the first to use the natural vegetation as medicine. Literary sources indicate the use of medicinal plants in ancient Assyria, Egypt and China, Circa back to 3000 B.C. in the early years of common era in Iran, Greece and Rome and during the middle ages in Arab countries, Middle Asia, Georgia, Armenia and Europe.

India has record achievements in the field of medicine and drugs. The use of medicinal plants as therapeutic agents can be traced in Atharvaveda, a religious book of Hindus, composed some 4000-5000 years ago. The prevalent use of medicinal plants at that time can be gauged by the fact that more than 2000 medicinal plants find special mention in this book. The traditional but ancient Indian System of Medicine, Ayurveda, has its origin in the days of Rig-Veda, which is one of the oldest repositories of recorded human knowledge known to the world. Ayurvedic system of medicine was developed into an elaborate science as is evident from the ancient Indian medicinal literature, Charaka and Susruta Samhita, written not latter than 1000 B.C. In addition, description of medicinal plants is also available in Brahma literature[1].

1.2. Bioactivity guided fractionation of medicinal plant:

After collection and proper identification by a professional taxonomist the plant is subjected to drying at ambient temperature in a shady place or in an oven with controlled airflow and temperature. The dried or stabilized plant material is powdered and subjected to a suitable extraction process as per standard operating procedures. The extracts are subjected to standard chromatographic techniques of fractionation and isolation of bioactive molecules as shown in Figure-1.

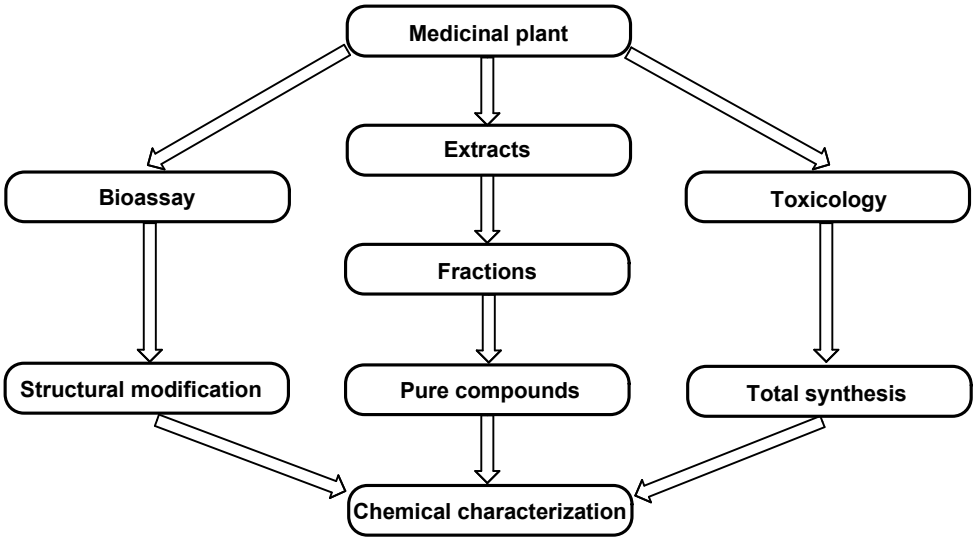


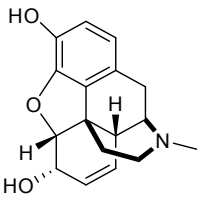
Figure 1. Methods for obtaining active substances from plants

This strategy is called bioactivity-guided fractionation. Bioassays can be performed using microorganisms, molluscs, insects, cellular systems (enzymes, receptors, etc), cell culture (animal and human), and isolated organs or *in vivo* (mammals, amphibians, birds, etc) [2-3]

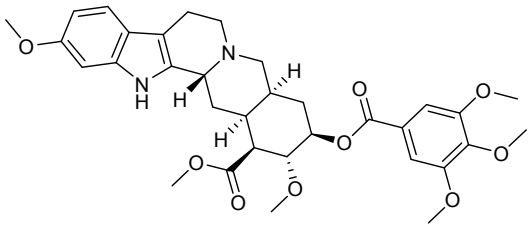
1.3. Secondary metabolites of plant origin:

The therapeutic properties of medicinal plants are conditioned by the presence, in their various parts, of active substances such as alkaloids, flavonoids, glycosides, steroids, terpenoides, tannins, coumarins, carotenoids, xanthones, iridoides and withanolides etc. As the scientific and technological knowledge developed and various attempts to probe into the architecture and behavior of the plant ingredients were made many pharmacologically lead compounds were discovered. Indeed, it is the architecture of these lead compounds whose duplication in organic laboratories has led to the development of basic and advanced organic chemistry. A number of medicinal plants when subjected to detailed chemical investigation involving various separation and characterization techniques led to isolation and identification of pure molecules which have been pharmacologically evaluated and as a result new drugs have been discovered, along with new applications. These bioactive molecules either

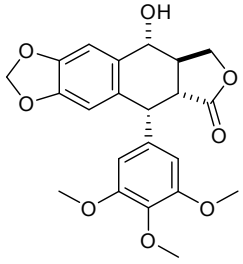
alone or in combinations are utilized as therapeutic agents or starting material for the synthesis of drugs or models for the synthesis of pharmacologically active compounds or new reagents for molecular biology research. Many of these molecules provide new and important leads against various diseases such as cancer, AIDS, malaria, cardiovascular disorders, typhoid and neurodegenerative diseases. Some of the important potential lead molecules like Podophyllotoxin, Camptothecin, Taxol, Vincristine and Vinblastine are purely anti-cancerous molecules of plant origin. Presently there are more than 125 clinically useful drugs of known constitution in the market which owe their existence to occurrence in plants. Some of the representative bioactive compounds isolated from the plant origin are shown as under.



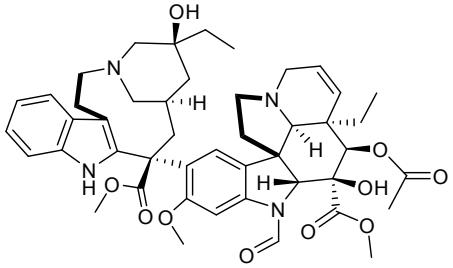
Morphine



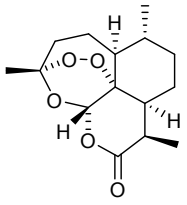
Reserpine



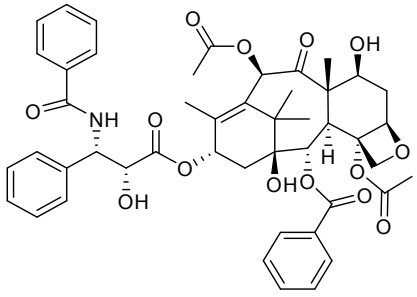
Podophyllotoxin



Vincristine



Artemisinin



Taxol

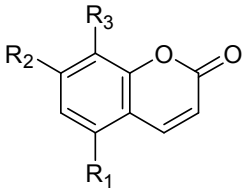
India has unique position in the world where traditional system of medicine is practiced since early times, Ayurveda being the oldest one. In addition to Ayurveda, Siddha, Unani, homeopathy and naturopathy tibetian system of medicine are regularly practiced for health care. These systems are plant based. Ayurveda contributes 85% followed by homeopathy (8%), Siddha (4%) and Unani (3.5%). Medicinal plants are utilized in traditional system of medicine, over the counter (OTC) non prescription items involving plant parts, extracts, galenicals and phyto-pharmaceuticals. Phytochemical investigations of various medicinal plants have led to the identification of quite a large number of compounds which have been classified into few broad classes with each class having its unique structural identity giving rise to a particular therapeutic importance. Various classes of compounds with their pharmacological importance have been listed as under-

1.3.1. Terpenoids:

This represents the major class, almost ubiquitous and chemically interesting class of natural products having a carbon frame work comprised of five carbon isoprene units. Terpenoid molecules are important for plant survival and possess chemical and biological properties that are beneficial to humans. They show immunomodulatory activity, inhibiting the proliferation of activated T-cells[5]. These bioactive constituents exert gastric ulcer healing activity as well as protection of the gastric mucosa. Low molecular weight sesquiterpenes stimulate local mucus synthesis[4] and prostaglandin production, by the gastric mucosa. They also stimulate or inhibit the phagocytosis. They are also known to have antioxidant and antiviral activities.[6-7] Most of the triterpenoid aglycones and saponins have anti-edema activity and hypoglycemic activity which makes these compounds as drugs of choice for the treatment of obesity and arthritis. Terpenoids from edible legumes and soybeans show anti-cancer activity.[8] Terpenoids also act as anti-HIV-1 agents and as insect antifeedants. They are also known to possess anti-diabetic[9] and anti-neoplastic activities.[10]

1.3.2. Coumarins:

These are naturally occurring benzopyrones widely distributed in umbelliferous plants.[11] The pharmacological, biochemical and therapeutic applications of simple coumarins depend upon the pattern of substitution. Coumarins are used in perfumes, deodorants, soaps, tobacco as flavouring agent and beverages. Coumarins can occur either free or combined with the sugar and can exhibit blood- thinning,[9] fungicidal,[12] and antitumor activities.[13] More complex compounds based on the coumarin nucleus include the dicoumarol/warfarin anticoagulants, aflatoxins and the psolarens (photosensitising agents). Coumarins have been used against elephantiasis[14]. These have been also used as vasorelaxants[15-17] and hepatoprotective.[18]Some of the representative coumarins are as under-

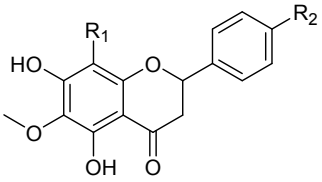


R_1 = H, R_2 = OGlc, R_3 = OH; Daphnin
R_1 = H, R_2 = OH, R_3 = OH; Daphnetin
R_1 = H, R_2 = OH, R_3 = OGlc; Daphnetin glucoside
R_1 = OGal, R_2 = H, R_3 = OH; Rhodonetin
R_1 = H, R_2 = Opentose, R_3 = OMe; Rhodonin
R_1 = H, R_2 = OH, R_3 = H; Umbelliferone

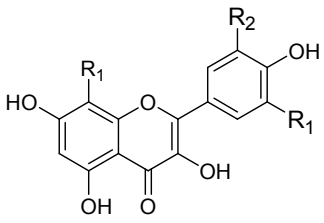
1.3.3. Flavonoids:

These represent a large family of low molecular weight polyphenolic secondary metabolites that are widely spread throughout the plant kingdom, ranging from mosses to angiosperms.[19] More than 6000 different flavonoids, including both aglycones as well as glycosides have been isolated from plants and the number is still increasing.[20] Flavonoids play important role as defence compounds and as signalling molecules in reproduction. These exhibit pathogenesis and symbiosis.[21-22] Flavonoids are known to have anti-inflammatory, [23] antiviral [24] and antimi crobial [25] activities. Flavonoids have been referred to as "nature's biological response modifiers" due to their inherent ability to modify the body's reaction to allergens, viruses and carcinogens.[26]Flavonoids from citrus plants especially hesperidin, quercetin and tangeritin possess antioxidant activity[27]

and an ability to increase intracellular level activity of vitamin-C. Hesperidin exerts beneficial effects on capillary permeability and blood flow.[28] Flavonoids play an important role in a variety of different intercations between plants and microorganisms, funtioning both as defence factors (phytoalexins) and as signalling molecules.[29,30] Some of the representative flavonoids are shown as under-



R_1= OMe, R_2 = OH; Farrerol
R_1= Me, R_2 = OMe; Matteucinol
R_1= H, R_2 = OH; 8-Desmethylfarrerol



R_1= R_2 = H; Kaemferol
R_1= H, R_2 = OH; Quercetin
R_1= R_2 = OH; Myricetin

1.3.4. Lignans:

Lignans comprise a class of natural products which are cinnamic acid derivatives and related biochemically to phenylalanine metabolism. Many lignans like Podophyllotoxin and its derivatives are known for physiological activity and represent drugs of choice for various kinds of cancer. Podophyllotoxin, isolated from *Podophyllum hexandrum* is a lignan of therapeutic importance well known for its antitumour activity. It acts as an inhibitor of assembly of microtubules and arrests the cell cycle in metaphase.[31-32]

1.3.5. Alkaloids:

These represent the metabolic by-products of amino acid origin and include an enormous number of bitter, nitrogenous compounds known for their bioactivities. Many alkaloids find applications as pharmacological ingredients. Ergotamine has been extensively used to relieve migraine headaches through the constriction of blood vessels. Hordenine is a potent phenyl ethylamine alkaloid with antibacterial and antibiotic properties in addition to its characteristic of helping in weight loss. Atropine, a tropane alkaloid is used as a cycloplegic, to temporarily paralyze the accommodation reflex[33] and as mydriatic; to dilate the pupils. Pepaverine, an opium alkaloid

used primarily in the treatment of visceral spasm, vasospasm especially those involving the heart and the brain, and occasionally in the treatment of erectile dysfunction. Opium and its other deravatives find usage as potent anaesthetics in addition to having narcotic properties.

1.3.6. Xanthones:

Biologically active plant phenols found in a few selected tropical plants. They are beneficial in many conditions of disorders including allergies, infections (microbial, fungal, viral),[34] high cholesterol level, inflammations, skin problems, gastrointestinal disorders and fatigue.[35] Xanthones have been found to support and enhance the body's immune system. These also exhibit strong antioxidant activity more potent than vitamin C as well as vitamin E. These are named as super antioxidants, which are beneficial for neutralizing free radicals in the body. Xanthones have been proved to possess beneficial effects on some cardiovascular diseases, including ischemic heart diseases, atherosclerosis, hypertension and thrombosis.

1.4. Natural products-need and perspectives:

The term "Natural products" covers an extremely large and diverse range of chemical compounds derived and isolated from biological sources. These natural products include entire organism such as plant, animal, or a microorganism that has not been subjected to any kind of processing or treatment other than a simple process of preservation. These can be even part of an organism such as leaves or flowers of a plant, an isolated animal organ. These also include an extract of an organism or part of an organism, and exudates. These can be pure compounds such as alkaloids, coumarins, flavonoids, glycosides, lignans, steroids, sugars, terpenoids, etc. isolated from plants, animals, or microorganisms However, in most cases the term natural products refers to secondary metabolites, small molecules (mol wt <2000 amu) produced by an organism that are not

necessarily for the survival of the organism.

Natural products played a prominent role in ancient traditional medicine systems, such as Chinese, Ayurveda, and Egyptian, which are still in common use today. According to the World Health Organization (WHO), 75% of people still rely on plant-based traditional medicines for primary health care globally. In recent years, a significant revival of interest in natural products as a potential source for new medicines has been observed among academia as well as pharmaceutical companies. According to a rough estimate 40% of the modern drugs in use have been developed from natural products. The use of natural products with therapeutic properties is as ancient as human civilisation and, for a long time, mineral, plant and animal products were the main source of drugs. The industrial revolution and the development of organic chemistry resulted in a preference for synthetic products for pharmacological treatment. The reasons for this were that pure compounds were easily synthesised with advent of modern techniques. Structural modifications to produce potentially more active and safer drugs can be easily performed and the economic power of the pharmaceutical companies is increasing.

However, even if we only consider the impact of the discovery of the penicillin, obtained from micro-organisms, on the development of anti-infection therapy, the importance of natural products is clearly enormous. About 25% of the drugs prescribed worldwide come from plants. Of the 252 drugs considered as basic and essential by the World Health Organization (WHO),11% are exclusively of p lant origin and a significant number are synthetic drugs obtained from natu ral precursor.

Interest in herbal drugs and natural medicine is undergoing a renaissance in the present age. Plant derived products represent around 25% of the total number of clinically used drugs. It has been reported that plants and other sources of natural products are superior sources of molecular diversity and molecular chemotypes, particularly in the areas where good synthetic leads do not exist. Despite competition from other

drug discovery methods, natural products are still providing their fair share of new clinical candidates and drugs. Between 1981 and 2002, 5% of the 1,031 new chemical entities approved as drugs by the US Food and Drug Administration (FDA) were natural products, and another 23% were natural product-derived molecules[36]as depicted below-

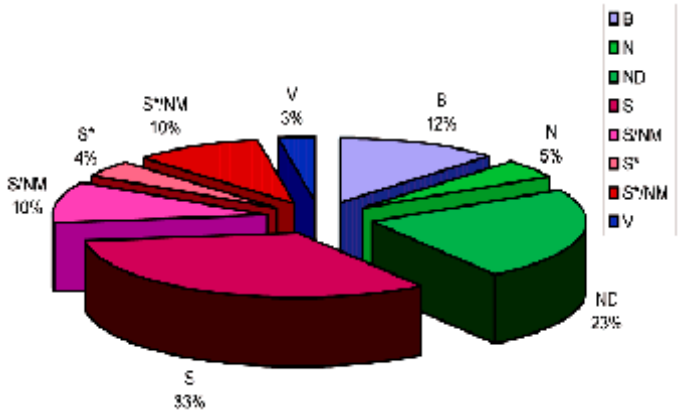


Figure 2.

"B": Biological; usually a large (>45 residues) peptide or protein either isolated from an organism/cell line or produced by biotechnological means in a surrogate host. **"N":** Natural product. **"ND":** Derived from a natural product and is usually a semisynthetic modification. **"S":** Totally synthetic drug, often found by random screening/modification of an existing agent. **"S*":** Made by total synthesis, but the pharmacophore is/was from a natural product.**"V":** Vaccine. **"NM":** Natural product mimic

About 25,0000 living plant species contain a much greater diversity of bioactive compounds than any chemical library made by humans.

1.5. Natural products and drug discovery:

Natural Products (NPs) traditionally have played an important role in drug discovery and were the basis of most early medicines [37–42]. Over the last 10 to 15 years advances in X-ray crystallography [43–45] and NMR [46–47] and alternative drug discovery methods such as rational drug design [48–50] and combinatorial chemistry [51–54] great pressure has been placed upon natural product drug discovery programmes and during this period most major pharmaceutical companies have terminated or considerably scaled down their natural product based operations[55–60]. However, despite the promise of

these alternative drug discovery methods, there is still a shortage of lead compounds progressing into clinical trials. This is especially the case in therapeutic areas such as oncology, immunosuppression and metabolic diseases where natural products have played a central role in lead discovery. In a recent review, Newman, Cragg and Snader analysed the number of natural product-derived drugs present in the total drug launches from 1981 to 2002 and found that natural products were a significant source of these new drugs, especially in the oncological and antihypertensive therapeutic areas[61]. In addition to providing many new drug leads, natural products and natural product-derived drugs were well represented in the top 35 worldwide selling ethical drugs in 2000, 2001 and 2002. [62]

Natural products have been the source of most of the active ingredients of medicines. This is widely accepted to be true when applied to drug discovery in 'olden times' when more than 80% of drug substances were natural products or inspired by a natural compound. Comparisons of the information presented on sources of new drugs from 1981 to 2007 indicate that almost half of the drugs approved since 1994 are based on natural products. Thirteen natural-product- related drugs were approved from 2005 to 2007, and as pointed out by Butler, five of these represented the first members of new classes of drugs which include the peptides as well.

Compounds from plants, microbes and animals, as well as synthetic or semi-synthetic compounds based on natural products cover a wide range of therapeutic indications such as anti-cancer, anti-infective, anti-diabetic, and show a great diversity of chemical structures. The chemical properties of small-molecule natural products have recently been developed into drugs and have been analysed. It is clear that, on average, natural products are more readily absorbed than synthetic drugs. Despite these advantages and the past successes, many large pharmaceutical companies have decreased the use of natural products in drug discovery screening. This has been because of the perceived disadvantages of natural products such as difficulties in access and supply, complexities of natural product chemistry and inherent slowness of working with natural products, and concerns about intellectual property rights, and the hopes associated with the use of collections of compounds prepared by combinatorial chemistry methods. Approximately 1

product-derived compounds are currently undergoing clinical trials and at least a 100 similar projects are in preclinical development (Table.1). Most of these compounds undergoing preclinical development are derived from leads from plants and microbial sources. The projects based on natural products are predominantly being studied for use in cancer or as anti-infectives, but many other therapeutic areas are also taken care off.

Table I. Drugs based on natural products at different stages of development

Development Stage	Plant	Bacterial	Fungal	Animal	Semi synthetic	Total
Preclinical	46	12	7	7	27	99
Phase I	14	5	0	3	8	30
Phase II	41	4	0	10	11	66
Phase III	5	4	0	4	13	26
Pre-registration	2	0	0	0	2	4
Total	108	25	7	24	61	225

Review of literature:

1.6. Bioactive constituents from related species of *Cotula anthemoides,* family-

Asteraceae:

The Asteraceae family comprises of some 23,000 plant species, the economical and medicinal importance of which has been widely described. An enormous variety of chemical classes have been isolated from members of the family, including monoterpenoids, sesquiterpenoids, sesquiterpene lactones, diterpenes, triterpenes, coumarins, flavonoids, polyacetylenes and benzofurans. Cotula is the largest genus of flowering plants found in the southern Hemisphere which belongs to family Asteraceae. It includes about 80 species of plants known generally as water buttons or button weeds.

Though sufficient reports are not available on *Cotula* anthemoides [63] but many species of the genus cotula have been worked out both for their phytochemical analysis as well as bioactivity evaluation. *Cotula cinerea,* commonly known as 'Al gartoufa', is used in Moroccan folk-medicine as an anti-inflammatory, analgesic, antiseptic and for the treatment of stomachache[64]. The extracts of this plant have been reported to show biological activities, such as antibacterial[65], analgesic[66] and molluscicidal[67]. Constituents such as flavonoids[68], sesquiterpene lactones[69] and sesquiterpene coumarins[70] have been isolated from this plant.

1.7. Brief introduction and phytochemistry of *Cotula anthemoides*:

Botanical description:

Family : *Asteraceae*

Sub-family : Asteroideae

Tribe : Anthemideae

Genus : Cotula

Species : *C. anthemoides*

Cotula anthemoides commonly known as 'Babuna' is a prostate or rising, pale-green, hairless annual herb with several, branches arising from the base upto 20 cm length. Leaves are nearly stalkless, ovate-oblong in shape, 1.5-5

cm (L) ×1.5-3.5 cm in dimensions and pinnately or bipinnately cut into narrowly oblong, toothed pointed segments. Flower-heads are almost spherical, yellow, 4-6cm across, solitary on thread like 4-5 cm long stalks. The cup holding the flower head is saucer shaped, with phyllaries narrowly oblong, 1-1.25×0.5-0.75 mm. Outer florets are female with an inconspicuous corolla, about half as long as those of disc-floret corollas. Babuna is a weed of gardens in the plains and lower altitudes in western Himalayas and is seen to undergo flowering in March-June. It is found in open spaces and is widely dispersed in warmer countries of the old world. The plant is weakly aromatic resembling *Anthemis cotula.* As far as its usage in traditional medicine is concerned, roots are taken in Lesotho for colic.[72-73] In South Africa it is a remedy for head and chest colds and is said to have been used extensively in the world wide flu-epidemic of 1919. Sometimes the leaf is crushed and snuffed into the nose.[70] People living in remote areas, mostly villagers soak this plant in hot water make a paste of it used for inflammation resulting mostly from fractures. In addition to its use for pulmonary and stomach troubles, it is mostly cultivated for ornamental purpose. The plant has occasionally been used in Europe as an aromatic lawn in substitution for Cammomile[73].

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Chapter -2

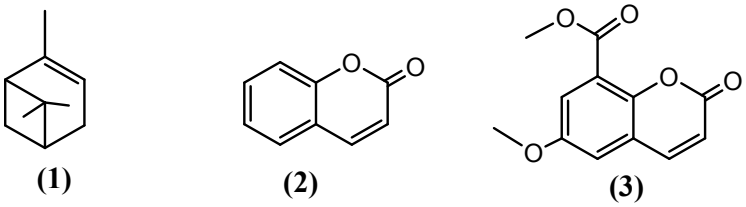
Results and discussion

2. Results and discussion:

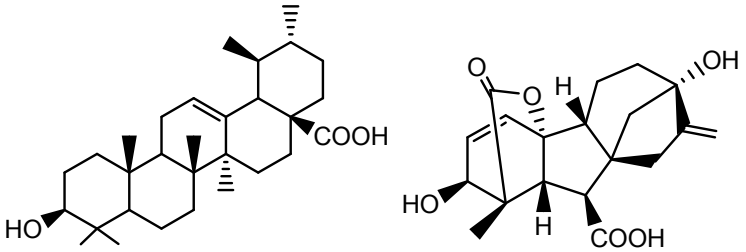
Natural products are reported to be evolutionary molecules with a profound impact on human health.[1-3] Nature's bio-synthetic engine produces innumerate secondary metabolites with distinct biological properties that makes them valuable as health products or as structural templates for drug discovery. Synthetic variation of structure and quantitative correlation of structure and activity (QSAR) are widely used tools in drug discovery[4]. A common finding in the natural product literature, in particular with regard to well established compounds, is that the same compound is reported to act on a myriad of targets with very different potential and with an inconsistent activity pattern, when subjected to tempering of its structure. This motive can be conceived by taking synthetic modification as a tool to make a particular compound to behave differently in different situations. Discovery and development of lead compounds from natural products has traditionally involved isolation of natural products with biochemical activity of interest, structural elucidation by various chemical and spectroscopic methods, development of chemical and biosynthetic methods for producing the compound and related compounds in larger quantities, structural modifications to produce lead structures and eventually the examination of struct ural activity relationships (SAR) and pharma-cological properties.

Taking cue from the nature and as part of our drug discovery programme, we aim to undertake a research programme in our institute to isolate and structurally modify the bioactive natural products in order to fine tune their biological potential and establish the SAR viz-a-viz structural features. In view of the importance of natural products and their derivatives, we have collected a high altitude medicinal plant named*Cotula* a*nthemoides* which belongs to the family *Asteraceae* from Kellar-Pulwama region of Kashmir valley. Identification of the plant material was done by Department of Plant Taxonomy, University of Kashmir and the Voucher Specimen **KASH-710** has been preserved in the Herbarium of University of Kashmir. Preliminary screening of various extracts (Hexane, Ethyl acetate, Methanol and aqueous) for their anticancer and antimicrobial potential confirmed only methanol extract to be significantly active in both the assays. In case of other extracts the activity was very mild. These results prompted us to go for the detailed phytochemical investigation of methanol extract and hence it was chromatographed on silica gel column (60-120 mesh) with Hexane – ethylaceate solvent system of variable

composition to yield α-Pinene (**1**), coumarin (**2**), a new substituted coumarin ester (**3**), Ursolic acid (**4**) andGibberlic acid-3 (**5**). Structural elucidation of these compounds was done by u sing^{1}HNMR,^{13}CNMR, IR, MS and 2D NMR techniques.



(1) **(2)** **(3)**

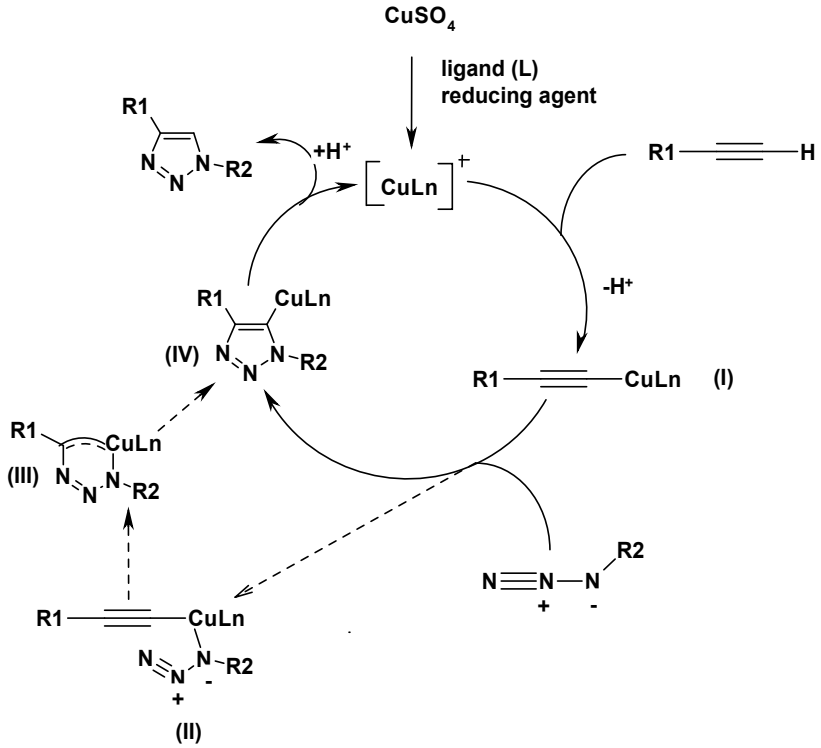


(4) **(5)**

Keeping in view the excellent biological activity of triterpenes such as ursolic acid, olenolic acid and betulinic acid, our work was directed towards the design and synthesis of lead compounds from ursolic acid through robust click chemistry approach. Accordingly a series of derivatives bearing bulky substituted triazoles was synthesized by Cu (I) catalyzed 1, 3-dipolar cycloaddition reaction of proparygylated ursolic acid derivative with different aromatic azides.

Click chemistry enables a modular approach to generate novel pharmacophores utilizing a collection of reliable chemical reactions which give products stereoselectively in high yields, produce inoffensive byproducts, are insensitive to oxygen and water, utilize readily available starting materials and have a thermodynamic driving force. Two types of click reactions that have influenced drug discovery are the nucleophilic opening of strained ring systems and 1,3-dipolar cycloadditions. Of particular interest is the copper catalyzed Huisgen [3+2] cycloaddition between a terminal alkyne and an azide to generate substituted 1,2,3-triazoles. This reaction is thought to proceed in a stepwise manner starting with the generation of copper (I) acetylide (**I**). Density functional

theory calculations show a preference for the stepwise addition (**I - II - III - IV**) over the concerted cycloaddition **(I-IV)** by approximately 12-15 kcal mol^{-1}, leading to the intriguing six membered metallocycle **III**.

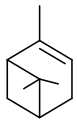


Postulated catalytic cycle for azide-alkyne coupling.

2.1. Characterization of compounds from *Cotula anthemoides:*

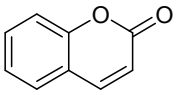
Compound 1: Compound **1** was obtained as transparent gummy liquid. ESI-MS showed the molecular ion peak atm/z 159 which corresponds to molecular formula $C_{10}H_{16}Na$, $[M+Na]^+$; IR spectrum showed absorption bands at: 3050, 2990,2920, 1480, 1450, 1410 cm^{-1}corresponding to C-H stretching, C-C single and double bond stretching respectively. Absence of absorption above 3200cm^{-1} confirms the absence of -OH or NH stretching. ^{1}H-NMR data δ 5.24 (1H, s, H-3), 2.40 (1H, t, J = 2.20, H-1), 2.18 (2H, t, J = 2.16, H-5), 2.00 (1H, t, J = 1.86, H-4) 1.92 (2H, dd, J = 2.1, H-7) 1.8 (3H, s, H-10), 1.4 (3H, s, H-8), 1.2 (3H, s, H-9) and ^{13}C-NMR with values at: 144.7 (C-2), 116.1 (C-3), 48.2 (C-1), 42.1 (C-5), 38.8 (C-7), 32.2 (C-4), 32.2 (C-6), 25.2 (C-9), 22.2 (C-8), 21.0 (C-10) confirmed the molecule as monoterpene. DEPT confirmed the presence of three C-H, two CH_2, three CH_3 leaving behind the scope for two quaternary carbons.

Based on these evidences, final structure of the compound was confirmed as α-Pinene. Comparison of its spectral data (IR, ^{1}H-NMR, ^{13}C-NMR and ESI-MS) with the literature values are also in agreement.



α-pinene (1)

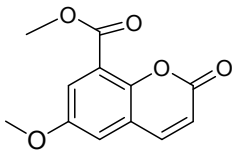
Compound 2: Compound **2** was recrystallized from pure chloroform as shining polyhedral crystals with melting point of 70^{o}C. Molecular ion peak in ESI-MS at m/z 169.2, $[M+ Na]^{+}$ correspond to the molecular formula $C_9H_6O_2Na$. Blue spot on TLC when visualized under UV light and absorption bands at (nm) : 217.6, 247.3, 257.4, 318.8 nm suggested a coumarin type structural framework. IR absorption bands at: 3040, 2955, 1690, 1620 cm^{-1}depicted the presence of a conjugated carbonyl in the compound; ^{1}H-NMR spectrum confirmed the presence of coumarin skeleton, showing two doublets one at δ 7.72 (1H, d, J = 8.0) and second at δ 6.42 (1H,d, J = 8.0) for two protons of double bond in lactone ring. Two mutiplets at δ 7.53 (2H, m) and 7.30 (2H, m) correspond to two sets of m-coupled protons in asymmetrically substituted benzene ring.^{13}C-NMR values of δ 160.7, 154.0, 143.5, 131.8, 127.9, 124.4, 118.8, 116.8, 116.6 represent lactone carbonyl oxygen attached aromatic carbons, other aromatic carbons and olefinic carbons.^{13}C-DEPT NMR showed the presence of three methines and three quaternary carbons. Comparison of its spectral data with data available in literature proved this to be coumarin with following structure.



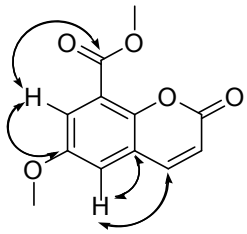
Coumarin (2)

Compound 3: Compound **3** was recrystallized from pure ethyl acetate as white crystalline needles; melting point 119-121^{o}C. ESI-MS of the compound showed molecular ion peak at m/z 257.2, $[M+ Na]^{+}$ which corresponds to the molecular formula $C_{12}H_{10}O_5Na$. Greenish blue spots on TLC when visualized under UV light along with absorption bands at (nm) : 219.4, 246.2, 261.6, 325.4 suggested the presence of coumarin nucleus in the molecule. IR absorption bands at: 3105,

1724, 1623, 1452, 1329 cm^{-1} represent C-H, ester carbonyl, enone carbonyl, C-O and C-C stretch respectively; ^{1}H-NMR spectrum confirmed the presence of coumarin skeleton, showing two doublets at δ value of 8.15 (1H, d, J = 12) and 6.60 (1H, d, J = 12) for two protons of double bond in lactone ring. Two doublets at δ value of 7.61 (1H, d, J = 2.4) and 7.39 (1H, d, J = 2.4) correspond to two m-coupled protons in asymmetrically substituted benzene ring. Two singlets at δ 4.08 (H, s), 3.97 (3H, s) correspond to two methoxy groups.^{13}C-NMR resonances at δ 165.2, 162.0, 150.3, 148.0, 143.6, 125.1, 123.2, 122.8, 119.4, 108.5, 55.9, 53.4 represent lactone carbonyl, ester carbonyl, oxygen attached aromatic carbons, other aromatic carbons and oxygen attached aliphatic carbons i.e., carbon atoms of methoxy groups.^{13}C-DEPT NMR showed the presence of four methines, six quaternary carbons and rest two carbons to be methoxy carbons. The position of substituents in the aromatic ring was confirmed by key HMBC connectivities. Hence, the structure of compound (**3**) was given as below:



6-Methoxy-2-oxo-2*H*-chromene-8-carboxylic acid methyl ester (3)

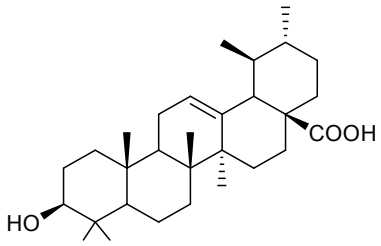


Key HMBC connectivities of **3**

Comparison of its spectral data with data available on structurally related coumarins proved this coumarin to be new to literature.

Compound 4: Compound **4** was obtained as white crystalline powder with melting point 283-285^{o}C. Absorption in UV(MeOH, nm) was observed at 224.6, 235.7, and 271.8. Absorption in IR at 3438 cm^{-1} showed the presence of hydroxyl group and absorption at 2930 cm^{-1} confirmed C-H stretch. Absorption at 1712 cm^{-1} and 1638 cm^{-1} showed the presence of carbonyl in the form of acid group and carbon-carbon double bond stretching. Stretching frequency values of 1452 and 1382 cm^{-1} confirmed C-O stretching vibrations. In ESI-MS mo

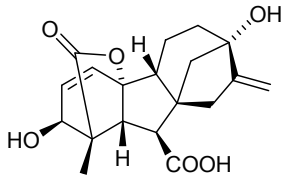
peak at *m/z* 456.5 $[M+Na]^+$ suggested the molecular formula $C_{30}H_{48}O_3Na$ for compound which in turn suggested a triterpenoid nucleus. In ^{1}H-NMR chemical shift, δ 5.13 (1H, s) correspond to vinylic proton at C-12, δ value of 3.49 (1H, d, *J* = 12.00) corresponds to proton at C- 3 which has attached hydroxyl group. Strong absorptions in the form of long peaks from δ 1.3-0.7 confirmed the presence of seven methyl groups. ^{13}C-NMR absorption peak at δ 178.4 showed the presence of carbonyl group in the form of acid. Two singnals at δ 138.2 and δ 124.6 confirmed the presence of olefinic carbon atoms in structure. Signal at δ 76.9 showed the presence of carbon attached to oxygen i.e., C-3. Presence of acid group was confirmed chemically by sodium bicarbonate test. ^{13}C-DEPT NMR confirmed the presence of six methines, seven methyls, nine CH_2 groups and finally leaving the scope for six quaternary carbons. Comparison of spectral data with the literature data suggested the molecule to a known cytotoxic pentacyclic triterpenoid, ursolic acid.



Ursolic acid (4)

Compound 5: Compound **5** was obtained as pale yellow powdery substance with melting point 230-233°C. $[\alpha]_D^{25}$ (c 0.50, MeOH) = +81.0° and UV absorption at (MeOH, λ_{max}, nm): 231.1, 245.6, 268.9. In IR spectrum absorption at (KBr, ν, cm^{-1}): 3447 cm^{-1} confirmed the presence of hydroxyl group in molecule. Presence of carbonyl in the form of acid was confirmed by absorption frequency at 1750 $cm^{-1}$and absorption at 1654 cm^{-1} showed the presence of carbon–carbon double bond. Molecular ion peak at m/z 385.1 $[M+K]^+$ confirmed by ESI-MS suggested the molecular formula $C_{19}H_{22}O_6K$ for the compound. In ^{1}H-NMR spectrum doublet at δ 6.41 (1H, d, *J* = 7.6) and double boublet at δ 5.92 (1H, dd, *J* = 7.6, 3.4) showed the presence of two olefinic protons located on two carbon atoms. One singlet at δ 5.24 (1H, s) corresponds to proton on carbon atom

between methyl and carboxyl substituted carbon atoms. Chemical shift values of 4.99 (1H, d, J = 16.40) and 4.24 (1H, d, J = 2.8) correspond to protons on carbon atoms substituted by carboxyl and alcohol group respectively. Absorption values in ^{13}C-NMR at chemical shift value of δ 183.6 and 177.8 correspond to presence of carbonyl in the form of acid and ester respectively. Resonances at δ values of 160.8, 136.6 and 135.8 represent olefinic carbon atoms. Similarly δ values of 95.1 and 81.2 represent carbon atoms to which acid and terminal of ester groups are attached. Absorption value of δ 73.09 represent quaternary carbon having hydroxyl attached. Presence of acid moiety was also confirmed chemically by sodium bicarbonate effervence test.^{13}C-DEPT NMR confirmed the presence of five CH_2 groups, four methine groups and one methyl group thus indirectly fixing the number of quaternary carbons to five

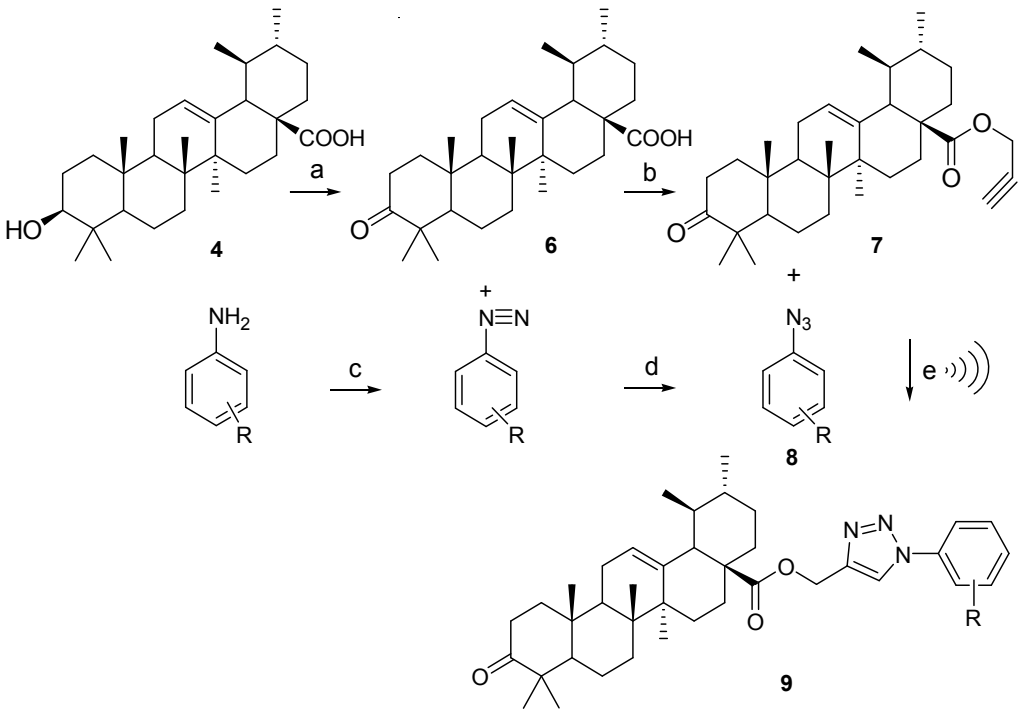


Gibberlic acid-3 (5)

2.2. Structural modifications of ursolic acid using click chemistry approach:

Keeping in view the available literature, concerning the biological activities of ursolic[5-12] acid and its analogues[13-15] along with the availability of sufficient amount of this compound it was convenient to go for synthetic modification and devise and synthesize a library of its modified analogues. Ursolic acid (**4**) was subjected to oxidation reaction using Jones oxidation conditions at 0 ^{o}C to result into the formation of C-3 oxidized ursolic acid derivative (**6**) in almost quantitative yield. Propargylation of the carboxylic group under Cs_2CO_3 condition in dry THF delivered the well poised terminal alkyne derivative of ursolic acid (**7**) in excellent yield. On the other hand aromatic azides (**8**) were prepared from their corresponding amines by diazotization with sodium nitrite in acidic conditions followed by displacement with sodium azide in good to

excellent yield. 1,3-dipolar cycloaddition reaction of **7** with various aromatic azides in presence of $CuSO_4.5H_2O$, sodium ascorbate in *t*-BuOH-H_2O (2:1) at 45^oC temperature under sonication resulted into the formation of ursolic acid triazole derivatives (**9**) in excellent yield (**Scheme-I**). All the structures were confimed by ^{1}HNMR, ^{13}C NMR, IR and ESI-MS analysis. In ^{1}HNMR cyclization of azides to form triazoles was confirmed by resonance of H-5 of triazole ring in aromatic region as well as by appearance of proton signals in aromatic region. The structure was further supported by the ^{13}CNMR and DEPT, which showed all the carbon signals corresponding to triazole derivatives. ESI-MS of all the compounds for $[M+Na]^+$ was also in quite agreement.



Scheme I. (a) CrO_3, H_2SO_4, acetone, 0 ^{0}C, 98%; **(b)** Propargyl bromide, Cs_2CO_3, THF, rt, 92%, **(c)** $NaNO_2$, H_2SO_4, dioxane, -10 ^{0}C, **(d)** aq. NaN_3, -15 ^{0}C (quantitaive yield over 2-steps); **(e)** Sodium ascorbate, CuSO4, *t*-BuOH: H_2O (2:1) at 45 0 ^{0}C, 72-98 %

Using the above 1,3-dipolar cycloaddition reaction of aromatic azides (**8**) with (**7**) in presence of $CuSO_4.5H_2O$ and sodium ascorbate, a series of triazole derivatives (**9**) differing in substitution at aromatic ring were preprared in good to excellent yield in a time span of 0.5 to 8 hours as shown in Table I.

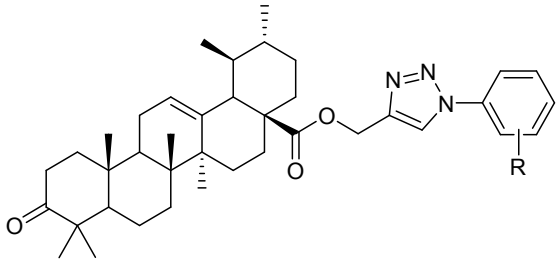
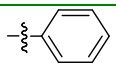
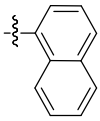
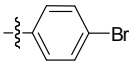
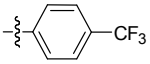
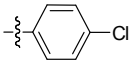
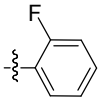
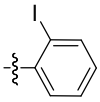
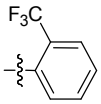
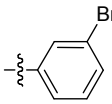
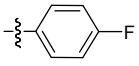
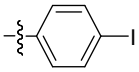
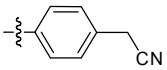


Table I. Ursolic acid triazolyl derivatives with various substitutions.

S.No.	Compound	R	Time(h). (min)	Yield (%)[a]
1	9a		08	89
2	9b		06	85
3	9c		05	96
4	9d		02	88
5	9e		08	85
6	9f		03	92
7	9g		0.5	98
8	9h		08	96
9	9i		0.3	95
10	9j		6.5	92
11	9k		0.4	96
12	9l		08	72

[a]Yields are reported after isolation of compounds

2.3. Biology:

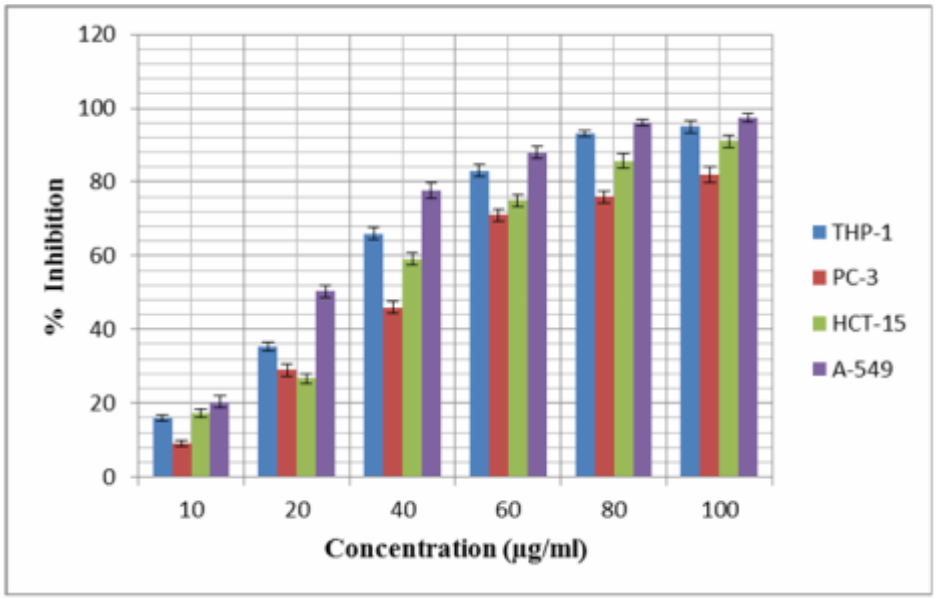
All the compounds including both isolated natural products and synthetically modified analogues were assayed for their *in vitro* cytotoxic activity againsta panel four different human cancer cell lines and anti-microbial activity against a set of seven bacterial and two fungal strains respectively.

2.3.1. Cytotoxic activity:

The cytotoxic activity of methanolic extract and isolated constituents thereof was studied against a panel of human cancer cell lines including THP-1 (leukaemia), A-549 (lung), PC-3 (Prostate) and HCT-15 (colon) by using sulphorhodamine-B assay.

Methanolic extract was found to be potentially active against all the tested cancer cell lines and it showed concentration dependent percentage growth inhibition with excellent activity at higher dose concentrations. These results are shown in Figure 1 and were made the base for further detailed investigations.

Figure 1: *In vitro* cytotoxic activity of the methanolic extract of *Cotula anthemoides* at different concentrations.

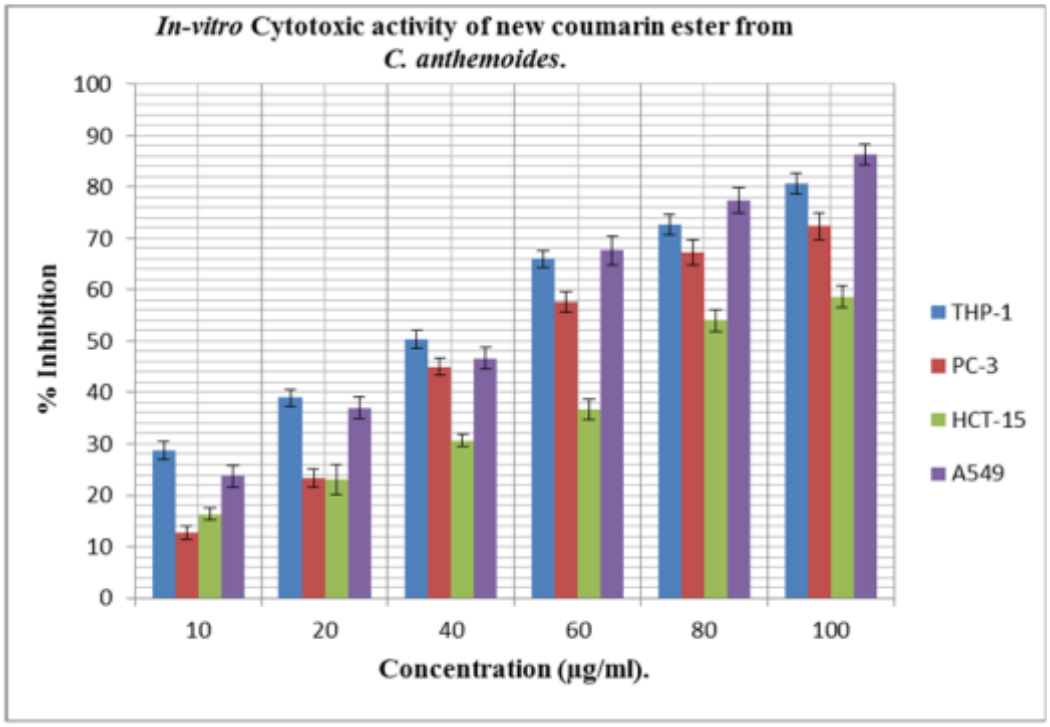


In case of the isolated constituents the cell lines were subjected to constant concentration of 50 µg/ml for 48 hours and percentage of dead cells was evaluated. The percentage of the dead cells in case of THP-1 (leukaemia), A-549 (lung), PC-3 (Prostate) and HCT-15 (colon) was found to be highest for compounds **(3)** and **(4)**. In order to evaluate the cytotoxic potential c

isolated coumarin methylester (**3**) in detail, different concentrations of this compound in the range of 10, 20, 40, 60, 80, 100 µg/ml were analysed against same cancer cell lines and it was found that compound (**3**) exhibited a dose-dependent inhibitory effect on all cell lines tested in the 10-100 µg/ml dilution range (Figure-2 & Table II).

Figure 2: *In vitro* cytotoxic activity of new coumarin ester from *Cotula anthemoides* at different

concentrations.



TableII. *In-vitro* cytotoxic activity of compound 1-5.

Tissue Type	Leukemia	Prostate	Colon	Lung
Cell line Type	**THP-1**	**PC-3**	**HCT-15**	**A549**
Comp.	**Conc. (µg/ml)**	**% Growth inhibition**		
1	50	26	35	28	43
2	50	63	58	51	40
3	10, 20, 40, 60,	28, 39, 52, 66, 73,	13, 24, 45, 58,	16, 24, 31, 37,	24, 38,47,

	80, 100	81	68, 73	55, 59	68, 78, 86
4	**50**	**86**	**91**	**77**	**79**
5	50	43	39	35	24
5-FU	$1x10^{-5}$M	32	45	78	48
Mito-C	$1x10^{-5}$M	23	69	31	51

5-FU = 5-flourouracil, Mito-C = Mitomycin-C

The IC_{50} values were found to be 40µg/ml (THP-1), 43 µg/ml (A-549), 76 µg/ml (HCT-15) and 47 µg/ml (PC- 3). Of the four cell lines that were tested, THP-1 (leukemia) and A-549 (lung) malignant cell lines were found to be the most susceptible.

Encouraged by the potential cytotoxic activity of compound (**3**) and (**4**) and with t he good abundance of compound (**4**) in hand, we shifted our attention towards its structural modifications. As a result, a battery of its triazolyl analogues were prepared and evaluated for anticancer activity against a panel of human cancer cell lines- A-549, THP-1, MCF-7, HCT-116 along with a normal cell line, FR-2 and the results are reported in Table III. Initially all the compounds were screened at 50µM concentration. All those derivatives which depicted significant activity at this very particular concentration were further screened at different lower concentrations for evaluation of their respective IC_{50} values (Table IV). From the percentage growth inhibition data and IC_{50} values (Tables III & IV) it is concluded that:

(a) Compounds **4**, **7**, **9a**, **9c**, **9g** and **9l** were found to be the most promising against all the tested cell lines. Results confirm the synthetically modified compounds to be better in activity than the parent ursolic acid (**4**)

(b) Among all the promising compounds, compounds **9a**, **9c** and **9g** were the most active in this study. Compound **9c** which contains the *p*- bromo substitution at aryl ring was found to be most promising with IC_{50} values in the range of 1-18 µM. It was observed from the data that this compound was specifically active against two cancer cell lines, THP-1 and HCT-116 with IC_{50} values of 1.0 and 1.9 respectively.

(c). From IC_{50} values, it is clear that almost all the active compounds exhibit less cytotoxic activity towards the normal epithelial cell line, FR-2 compared to their potential against cancer cell lines

Table III. ***In-vitro*** **cytotoxic activity of synthetically modified compounds.**

	Tissue		Lung	Breast	Colon	Leukemia	Normal cell line
	Cell Line		A-549	MCF-7	HCT-116	THP-1	FR-2
S. No	Code	Conc.(µM)					
01	**4**	50	61	57	53	84	66
		10	28	36	55	31	31
02	**7**	50	78	42	96	88	86
		10	50	22	74	42	33
03	**9a**	50	64	51	78	83	45
		10	43	28	44	54	12
	9b	50	27	40	02	47	ND[a]
04	**9c**	50	63	78	92	95	93
		10	45	44	80	74	85
05	**9d**	50	30	03	16	27	ND
	9e	50	26	52	49	09	ND
06	**9f**	50	18	15	12	49	ND
	9g	50	67	76	78	79	ND
07		10	43	54	44	56	
08	**9h**	50	46	04	23	73	ND
09	**9i**	50	16	03	10	32	ND
10	**9j**	50	03	04	03	02	ND
11	**9k**	50	01	08	08	01	ND
12	**9l**	50	26	34	67	55	46
13		10	13	17	44	32	14
14	5-FU[b]	20	86	NT	76	96	ND
15	Mito-C[c]	01	70	82	NT	NT	ND

a =Not determined,b = 5-flourouracil, c = Mitomycin-C

Table IV. IC_{50} of potential candidates.

Tissue Type		**Lung**	**Leukemia**	**Colon**	**Breast**
Cell Line Type		**A-549**	**THP-1**	**HCT-116**	**MCF-7**
S.No.	**Code**	**IC_{50} Values in (µM)**			
1	**4**	33	9.1	42	37
2	**7**	15	13	2.3	56
3	**9a**	24	11	14	39
4	**9c**	**18**	**1.0**	**1.7**	**15**
5	**9g**	19	9.4	15	13
6	**9l**	>100	37	19	79

2.3.2. Antibacterial activities.

Antimicrobial activity of all compounds was evaluated against a set of seven bacterial strains (including both Gram-positive and Gram-negative) and two fungal strains and their potency was assessed by measuring the inhibition zones, MIC and MBC/MFC values. The results (MICs and MBC/MFC) indicate that chemical constituents displayed a broad spectrum and variable degree of antibacterial activity against the different tested strains. The most susceptible bacterial strain incase of known and new constituents (Table V) was found to be *Staphylococcus aureus* (MIC=64µg/ml, MBC >64 µg/ml) and *Bacillus subtillis* (MIC=64µg/ml, MBC >128 µg/ml) respectively. Concerning the Gram negative b acteria, isolates demonstrated a significant activity especially against *Shigella dyssenteriae* (MIC=64 µg/ml). Some Gram-positive bacterial strains exhibited lower MIC/MBC values than Gram-negative bacteria thereby indicating that isolates are more active against Gram-positive bacteria as compared to Gram-negative bacteria.

Table V. Antibacterial activities of compounds **1-5**.

Compound	*B. subtillis*	*S. epidermidis*	*P. aeruginosa*	*S. aureus*	*S. typhi*	*S. dyssenteriae*	*K. pneumonia*	MIC	MBC
	Diameter of inhibition zone (mm)								
1	24.3	16.6	21.5	22.6	19.6	19.1	15.6	>64, >128, >64, >32, 128, >128, >128	>128, 256, >128, >64, >128, 256, 256
2	19.0	17.5	19.9	17.3	16.6	20.0	14.8	128, >128, 128, 256, >256, >128, >256	256, >256, 256, >256, 512, 256, >512
3	26.1	19.6	23.3	25.1	18.3	22.2	17.9	>64, >128, 128, 128, >128, 128, >128	>128, 256, >128 , >128, 256, >128, 256
4	23.7	22.8	21.7	25.6	20.7	23.3	18.3	>64, 128, 128, 64, 128, 64, >128	128, >128, >128 , >64, >128, >64 , >128
5	18.3	20.7	19.7	15.9	17.6	19.3	16.2	>128, 128, >128, 256, >128, 128, 256	256, >128, 256, >256, 256, 256, >256
AMP	36.6	38.0	34.9	37.6	33.7	39.6	39.5	8, >4, >8, >4, >8, 4, 4	16, 8, >16, 8, 16, >4 >4
VAN	34.5	36.2	33.6	37.8	32.1	38.9	36.6	>8, 8, >8, >4, 16, >4, 8	16, 16, >16, 8, >16, 8, >8

AMP = Ampicillin (5µg disc) andVAN = Vancomycin

The two fungal strains viz., *Penicillium chrysogenum* and *Aspergillus niger* were also susceptible to isolated constituents showing MIC/MFC = 8 µg/ml 16 µg/ml respectively (Table VI).

Table VI. Antifungal activities of compounds **1-5**.

Compound	*P. chrysogenum*	*A.niger*	MIC	MFC
	Zone of inhibition (mm)			
1	07.40	11.60	64, 32	128 , >32
2	80.20	09.90	>64, >64	>128, 128
3	**09.15**	**10.65**	**>32, >32**	**128, 64**
4	**12.24**	**14.34**	**>8, 8**	**>16, 16**

5	06.85	08.22	128, 64	256, 128
Fluconazole	13.20	19.60	>4, 4	8, >4

In case of synthetically modified compounds the results of antimicrobial activity (Table VII & VIII) were very interesting-

(a) Increase in hydrophobicity in case of **9a** and **9b** resulted in increase of antibacterial potential compared to parent compound (**4)** but this increase was very low.

(b) Significant increase in antibacterial activity in case of compounds **9d**, **9f**, **9g**, **9h**, **9j**, **9k** and antifungal activity in case of compounds (**7)**, **9b**, **9i**, and **9l** can be accessed from their respective MIC and MBC/MFC values. All these compounds which showed enhanced activity contain hetero atoms fluorine and iodine at aryl ring site except compound **9b.**

(c) Compounds **9d**, **9j** and **9k** were most potent antibacterial analogues out of the whole series with enhanced activity compared to parent compound (**4).**

(d) Tremendous increase in antifungal activity was seen in case of compounds **9b** and **9i**.

Table: VII. Antibacterial activities of synthetically modified compounds**.**

Compound	*B. subtillis*	*S. epidermidis*	*P. aeruginosa*	*S. aureus*	*S. typhi*	*S. dyssenteriae*	*K. pneumonia*	MIC	MBC
Zone of inhibition(mm)									
4	23.70	22.80	21.70	25.62	21.75	23.35	18.38	>64,128, 128, 64, 128, 64, >128	128,>128, >128,>64, >128, >64, >128

7	17.45	16.20	17.75	19.25	15.90	15.40	13.85	>128,>128, 128, >64,256, 256, >256	256,>256,>128, 128, >256,256, 512
9a	22.60	23.55	16.30	21.70	12.65	12.00	10.50	>64, >64,>128, 128, 256,>128, 256	>128, >128, 256, 128, >256, >256, >512
9b	25.35	24.90	15.45	26.95	10.70	11.80	09.00	64, >64, 256, 64, >256, 256, 512	>64, 128, >256, >64, >512, 512,>512
9c	18.85	15.60	19.30	21.80	19.00	21.55	16.90	128,>128, 128, 64, >128, 128, >256	>128,>256, >128,128, 256,>128 ,256
9d	31.45	33.20	31.15	33.40	27.25	32.60	25.45	>16, 16, 16,>8, 32, 16, 32	>32, 32, 32, 16, >32, >16, 64
9e	17.80	19.35	17.00	17.80	14.00	16.65	13.00	>128,128,128, 128,>256,256, >256	>256,>128, >128,128, 512,256,512
9f	25.50	27.35	27.00	31.45	25.90	29.75	22.60	>32, 64, >64, >16, >32, 32, >32	64, >64, 128, >32, >64, >32, >64
9g	26.40	29.00	25.55	33.00	28.70	31.00	27.65	32, >32, >64, 16, 32, 32, >16	64, 64, 128, 32, 64, >32, 32
9h	24.75	30.25	28.70	26.50	30.15	27.70	23.90	>32, 32, 64,>16, 32, >32,32	64, >32, >64, >32,>32,64, >32
9i	14.95	15.55	12.00	16.45	11.50	12.90	08.40	256, 256, >256,>128, 256, 256, 512	>256, >256, 512, >256, 512, >256, >512
9j	34.80	31.45	33.15	35.70	32.10	34.15	27.65	8, >16, 16, 8, 16, >4, >16	16, >32, 32, >8, >16, 8,>32
9k	32.65	30.00	29.00	34.20	31.00	25.95	28.60	16, >16, 32, 8, 16, >16, 16	>16, >32, 64, 16, >16, >32, 32.
9l	15.50	15.65	12.60	16.70	08.70	09.00	11.45	>256, 256, 256,>128, >256,512, >256	512, >256, 512, >256, 512, >512, 512

Table: VIII. Antifungal activities of synthetically modified compounds**.**

Compound	*P. chrysogenum*	*A.niger*	MIC	MBC
		Zone of inhibition (mm)		
4	12.30	14.34	>8,8	>16, 16
7	16.50	21.75	4, >4	>8, 8
9a	17.20	15.70	4, >8	>8, 16
9b	19.80	20.35	4, 4	8, >4
9c	14.60	17.78	8, >8	>16, >16
9d	10.50	09.10	16, >16	>32, 64
9e	12.00	11.70	8, >8	16, >16
9f	09.85	12.65	32, >16	64, >32
9g	10.10	14.20	>32, >8	64, 16
9h	14.40	12.75	8, >8	16, 32

9i	20.40	22.90	4, 4	8, >4
9j	09.00	15.60	>32, >8	64, >16
9k	10.80	10.25	>16, >32	32, >64
9l	16.90	19.30	>4, 4	>8, 8
Fluconazole	13.20	19.62	>4, 4	8, >4

Conclusion:

To sum up a bioassay guided fractionation protocol for methanolic extract of *Cotula anthemoides* resulted in the isolation of one new and four known chemical constituents, all reported for the first time from this plant. The chemical constituents were characterized by making use of various spectroscopic techniques like ^{1}HNMR,^{13}CNMR, HMBC, IR and ESI-MS . All these compounds were assayed for their cytotoxic and antimicrobial potential against a panel of human cancer cell lines and various bacterial and fungal strains. Compounds **3** and **4** were found to be significantly active in both the tested assays. On the basis of potential biological activity of compound **4** along with its good quantity in hand, it was further subjected to structural modifications to alter its biological potential. As a result, a series of ursolic acid-triazolyl derivatives were synthesized by click chemistry approach. All the derivatives were screened for their anticancer and antimicrobial activity. Anticancer activity was tested against a panel of four human cancer cell lines including A-549, THP-1, MCF-7, HCT-116 along with a normal epithelial cell line, FR-2. Antimicrobial activity was evaluated against seven bacteria and two fungal strains. From the anticancer screening data, it was interesting to note that some of the compounds exhibited interresting anti-cancer activity. Compound **9c** which contains the *p*-bromo substitution at aryl ring was found to be most promising with a 2-25 fold decrease in its IC_{50} value. The results of antimicrobial screening (MICs and MBC/MFC) indicate that these synthesised compounds displayed a broad spectrum and variable degree of antibacterial and antifungal activity against the different tested strains. Compounds **9d**, **9j** and **9k** were most promising antibacterial analogues and compounds **9b** and **9i** were found to be most promising antifungal analogues in this study.

2. 4. Refrences:

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Chapter -3

Experimental

3.1 General:

Melting points were determined on digital Buchi B-545 melting point apparatus using glass capillaries and are uncorrected. Infrared spectra were recorded as cm^{-1} absorption using KBr pellet method on Perkin Elmer-FT-IR spectrometer. Optical rotations were measured in Jasco DIP-360 digital polarimeter. Ultraviolet (UV) spectra's were recorded in methanol on Specard S 100. ^{1}H NMR and ^{13}C NMR, COSY, HMBC, HSQC were recorded on 500, 400 and 100 MHz Bruker Daltonics spectrometers. The chemical shifts (δ) are reported in ppm relative to tetramethylsilane (TMS) as internal standard and coupling constants were measured in Hz. Mass spectra were recorded on Bruker Daltonics electro spray ionization apparatus.

Column chromatography was carried out on silica gel (Qualigens, 60-120 mesh) in a glass column with diameter 7.0 cm and length 90 cm. Pre-coated silica gel thin layer chromatographic (TLC) plates were viewed with ultraviolet light at 254 nm for fluorescence quenching spots and at 366 nm for fluorescent spots. Cerric sulphate

, sulphuric acid, anisaldehyde, vanillin and $FeCl_3$ were used as spraying reagents to visualize different spots.

3.2 Plant material:

The aerial parts of plant *Cotula anthemoides* were collected from the hilly area of Kellar- Pulwama in the month of May 2011 and after proper identification by Dr. A. R. Naqshi (Professor of Plant Taxonomy, University of Kashmir) a Voucher Specimen **KASH-710** was submitted to the same department for records.

3.3 Extraction and isolation:

Shade dried and coarsely powdered aerial parts of plant material (1.5kg) were subjected to defatting with petroleum ether for 48×3 hours at room temperature. Defatted plant material was dried further and extracted with methanol for 48×3 hours at room temperature. Evaporation of the solvent under reduced pressure delivered 65 gm of methanolic extract. This extract was dissolved in minimum amount of methanol, mixed with silica gel with mesh-size 60-120 and processed to result in fine and granular slurry. The dried slurry was loaded on a glass column of diameter 7.0 cm and length 90 cm and column chromatographed on silica gel with hexane, ethylacetate and methanol solvent systems of variable composition (Pure hexane, 5, 10, 15 % hexane - ethyl acetate etc.). Column elution resulted in collection of following fractions (100ml each): 1-75 (pure hexane, fraction-A), 75-170 (hexane – ethylacetate 95:05, fraction B) 170-310 (hexane - ethyl acetate 90:10, fraction-C), 310-400 (hexane - ethylacetate 85:15, fraction-D) 400-475 (hexane - ethyl-acetate 80:20, fraction-E) 475-600 (hexane - ethylacetate 75:25, fraction F) 600-710 (hexane - ethylacetate 65:35, fraction-G) 710-850 (hexane - ethylacetate 55:45, fraction-H) 850-1000 (hexane - ethyl-acetate 45:55, fraction-I) 1000-1150 (ethyl-acetate - methanol95:05, fraction-J) 1150-1310 (ethylacetate - methanol 90:10, fraction-K) 1310-1400 (ethylacetate - methanol 80:20, fraction-L) 1400-1550 (ethyl-acetate - methanol 60:40, fraction-M) 1550-onwards (pure methanol, fraction N).

Fraction B on concentration under reduced pressure resulted in compound **1** which was obtained as pungent smelling transparent gum like mass and was purified by filtration through a silica gel patch. Fraction-C on evaporation and recrystallization from ethylacetate afforded compound **2**. Fraction-D upon concentration under reduced pressure resulted in precipitation of compound **3**

which was also present in fraction- C but in very low concentration. Fraction F and G resulted in separation of compound **4** as yellowish impure powder. It was subjected to continued solvent treatment (hexane: chloroform, 97:03) for purification and white fine granular powder was obtained which gave a single pinkish spot on TLC when charred with ceric sulphate. Compound **5** got precipitated as yellowish powder from fraction-L on evaporation under reduced pressure and finally it was recrystallized from Isobutanol and water (95:05) after 96 hours.

Compound 1: Transparent gummy liquid, R_f = 0.52 ($CHCl_3$ - Hexane, 70:30 v/v), $[\alpha]_D^{25}$ (*c* 0.25, MeOH): -37 ; UV (MeOH, λ_{max}, nm): 216, 229.2; IR (KBr, ν, cm^{-1}): 3050, 2990, 2920, 1480, 1450 1410; ESI-MS: *m/z* 159.02 $[M+Na]^+$, (calculated for $C_{10}H_{16}Na$); ^{1}H-NMR (400 MHz, $CDCl_3$, δ, ppm, *J*/Hz): 5.24 (1H, s, H-3), 2.40 (1H, t, *J* = 2.20, H-1), 2.18 (2H, t, *J* = 2.16, H-5), 2.00 (1H, t, *J* = 1.86, H-4), 1.92 (2H, dd, *J* = 2.1, H-7), 1.8 (3H, s, H-10), 1.4 (3H, s, H-8), 1.2 (3H, s, H-9); ^{13}C-NMR (100 MHz, $CDCl_3$, δ, ppm): 144.7 (C-2), 116.1 (C-3), 48.2 (C-1), 42.1 (C-5), 38.8 (C-7), 32.2 (C-4), 32.2 (C-6), 25.2 (C-9), 22.2 (C-8), 21.0 (C-10).

Compound 2: Transparent crystalline compound, recrystallized from chloroform ; melting point 70°C; R_f : 0.40 ($CHCl_3$- Hexane, 1:1 v/v); IR (KBr, ν, cm^{-1}): 3040, 2955, 1690, 1620, 1480; ESI-MS: *m/z,* 169.2 $[M+Na]^+$, (calculated for $C_9H_6O_2Na$); ^{1}H-NMR (400 MHz, $CDCl_3$, δ, ppm, *J*/Hz): 7.72 (1H, d, *J* = 8.0), 7.61 (1H, d, *J* = 2.4), 7.53 (2H, m), 7.30 (2H, m), 6.42 (1H, d, *J* = 8.0); ^{13}C-NMR (100 MHz, $CDCl_3$, δ, ppm): 160.7, 154.0, 143.5, 131.8, 127.9, 124.4, 118.8, 116.8, 116.6.

Compound 3: Fine white crystalline substance, recrystallized from pure ethylacetate; melting point 119-121°C; R_f: 0.48 ($CHCl_3$ - Hexane, 1:1 v/v); $[\alpha]_D^{25}$ (*c* 0.40, MeOH): -59°; UV (MeOH, λ_{max}, nm): 236.4, 246.2, 261.6; IR (KBr, ν, cm^{-1}): 3105, 3017, 2970, 1724, 1623, 1452, 1329; ESI-MS: *m/z,* 257.20 $[M+Na]^+$, (calculated for $C_{12}H_{10}O_5Na$); ^{1}H-NMR (400 MHz, $CDCl_3$, δ, ppm, *J*/Hz): 8.15 (2H, m), 7.61 (1H, d, *J* = 2.4), 7.39 (1H, d, *J* = 12.0), 4.08 (3H, s), 3.97 (3H, s); ^{13}C-NMR (100 MHz, $CDCl_3$, δ, ppm): 165.2, 161.9, 150.3, 147.9, 143.6, 125.1, 123.2, 122.8, 119.4, 108.5, 55.8, 53.4.

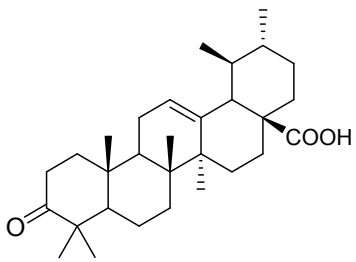
Compound 4: White crystalline powder ; melting point 283-285 ^{o}C; R_f: 0.62 (Hexane - EtOAc, 1:1 v/v); $[\alpha]_D^{25}$ (c 0.30, MeOH) = +59^{o}; UV (MeOH, λ_{max}, nm): 244.60, 255.72, 271.80; IR (KBr, ν, cm^{-1}): 3438, 3403, 2930, 1689, 1658, 1452, 1382, 1242, 1040; ESI-MS: *m/z,* 456.50 $[M+Na]^+$, (calculated for $C_{30}H_{48}O_3Na$); ^{1}H-NMR (400 MHz, $CDCl_3$, δ, ppm, *J*/Hz) : 5.13 (1H, s), 3.49 (1H, d, *J* = 12.0), 3.01 (1H, s), 2.51 (1H, s), 2.10 (1H, d, *J* = 8.0), 1.86 (3H, m), 1.41 (12H, m), 0.84 (21H, m); ^{13}C-NMR (100 MHz, $CDCl_3$, δ, ppm): 178.4, 138.2, 124.6, 76.9, 54.8, 52.4, 47.1, 46.9, 41.7, 39.9, 39.76, 39.7, 39.6, 39.4, 39.3, 39.1, 39.1, 38.9, 38.5, 38.4, 36.5, 28.3, 23.3, 21.1, 17.1, 16.9, 16.1, 15.3.

Compound 5: Pale-yellow powdery substance; melting point 230-233 ^{o}C; R_f: 0.55 (MeOH - EtOAc, 3:7v/v); $[\alpha]_D^{25}$ (c 0.50, MeOH) = +81.0^{o}; UV (MeOH, λ_{max}, nm): 231.12, 245.66, 268.90; IR (KBr, ν, cm^{-1}): 3447, 3373, 2969, 2934, 2877, 1750, 1654, 1483,1448, 1329, 1263, 1171; ESI-MS: *m/z,* 385.10 $[M+K]^+$, (calculated for $C_{19}H_{22}O_6K$); ^{1}H-NMR (400 MHz, $CDCl_3$, δ, ppm, *J*/Hz) : 6.12 (1H, d, *J* = 7.6), 5.72 (1H, dd, *J* = 7.6, 7.6), 5.08 (1H, s), 4.80 (1H, d, *J* = 16.4), 3.86 (1H, d, *J* = 2.8),3.18 (1H, t, *J* = 1.2), 3.08 (1H, d, *J* = 8.8), 2.54 (1H, d, *J* = 8.4), 2.22 (1H, d, *J* = 12.40), 2.10 (1H, d, *J* = 12.40), 1.85(2H, m) 1.72 (2H, dd, *J* = 10, 8.8) 1.65 (2H, d, *J* = 8.4) 1.60 (2H, m) 1.22 (3H, s); ^{13}C-NMR (100 MHz, $CDCl_3$, δ, ppm): 183.6, 177.8, 160.8, 136.6, 135.8, 110.1, 95.06, 81.1, 73.1, 57.5, 56.5, 54.9, 54.7, 53.80, 48.1, 46.7, 42.3, 20.5, 17.5.

3.4. Experimental:

3.4.1. Synthesis of compound 6:

To a solution of ursolic acid (400 mg, 0.88 mmol) in acetone (9 ml) at 0^{o}C, two drops of Jones reagent was added and reaction mixture was stirred at room temperature. Reaction was monitored by TLC (3:7, ethylacetate - hexane) till its completion in around 2 hours. Water was added to the reaction mixture and extraction was done by ethylacetate. The product was purified by column chromatography to deliver the pure compound **6** (365 mg, 91% yield).



White solid; mp: 276°C

^{1}HNMR(400MHz,$CDCl_3$, δ): 5.29 (1H, s), 3.20 (1H, d, *J* = 12 Hz), 2.28 (2H, t, *J* = 4.2 Hz), 2.14 (2H, d, *J* = 2.8 Hz), 1.90 (3H, t, *J* = 6 Hz), 1.81 (H, m), 1.76 (1H, m), 1.56 (3H, s), 1.50 (6H, s), 1.48 (3H, s), 1.35 (3H, s), 1.10 (6H, s).

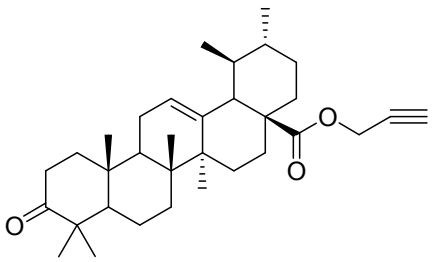
^{13}CNMR (100MHz, $CDCl_3$, δ): 216.6, 181.5, 151.9, 123.2, 53.6, 52.2, 47.8, 46.8, 41.7, 39. 9, 39.8, 39.7, 39.6, 39.4, 39.3, 39.1, 39.1, 38.9, 38.5, 38.4, 36.6, 28.3, 23.3, 21.2, 17.1, 17.0, 16.1, 15.3.

IR (KBr): 2930, 1718, 1657, 1456, 1378, 1242, 1042, 751.0 cm^{-1}

Mass (ESI-MS): 455.46 $[M+Na]^+$

3.4.2. Synthesis of compound 7:

To a solution of **6** (365 mg, 0.8 mmol) in THF was added cesium carbonate (521 mg, 1.6 mmol) and propargyl bromide (476 mg, 4 mmol) and the reaction mixture was stirred at room temperature for about 4 hours. Reaction was monitored by TLC (EtoAc- Hexane, 3:7 v/v) and the crude product was subjected to column chromatography to give pure compound **7** (356 mg, 98% yield).



White powdery solid; mp: 279 °C

^{1}HNMR (400 MHz, $CDCl_3$, δ): 5.30 (1H, m), 4.62 (2H, dd, *J* = 12, 12 Hz), 2.59 (1H, s), 2.54 (1H , m), 2.40 (2H, t, *J* = 4.2 Hz), 2.14 (2H, d, *J* = 2.8 Hz), 1.90 (3H, t, *J* = 6 Hz), 1.81 (1H, m), 1.76 (1H, m), 1.56 (3H, s), 1.50 (6H, s), 1.48 (3H, s), 1.35 (3H, s), 1.10 (6H, s).

^{13}C NMR (100 MHz, $CDCl_3$, δ): 217.9, 176.6, 137.9, 125.6, 78.1, 74.4, 55.3, 52.9, 51.6, 48.2, 47.4, 46.8, 42.2, 39.6, 39.3, 39.1, 38.8, 36.7, 36.4, 34.2, 26.6, 23.5, 23.4, 21.5, 21.1, 17.2, 17.0, 15.3.

IR (KBr): 2939, 2565, 2455, 2417, 1717, 1654, 1464, 1378, 1246, 1042, 779, 718 cm^{-1}

Mass (ESI-MS): 516.36 $[M+Na]^+$

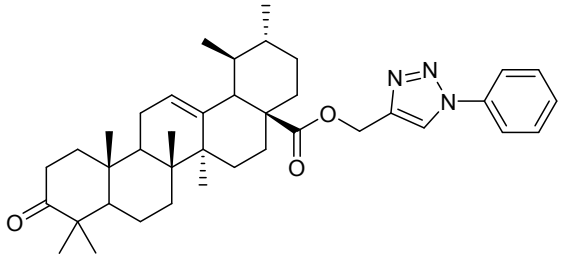
3.4.3. General procedure for the synthesis of 8:

To a solution of aromatic amine (1 equivalent) in 1, 4- dioxane at -15 ^{0}C, 5 equivalents of 2M Sulphuric acid was added in small installments while stirring. After 5 minutes 2 equivalents of 3M sodium nitrite solution was added drop wise and after 30 minutes 3 equivalents of 3M sodium azide solution was added carefully. Reaction mixture was brought to room temperature and extracted with diethyl ether. Organic layers were washed with saturated sodium bicarbonate solution and dried over anhydrous sodium sulphate and evaporation was done at reduced pressure at room temperature to deliver the pure azides in quantitative yield which were used for further reaction without purification.

3.4.4. General procedure for the synthesis of compounds 9a-9l:

To a solution of compound **7** (15 mg, 0.03 mmol) in *t*BuOH - H_2O (2:1, 5 ml) was added sodium ascorbate (1.2 mg, 0.006 mmol) and $CuSO_4$ (1.2 mg, 0.0045 mmol). To this mixture, aryl azide (2 equivalents) was added and the reaction mixture was sonicated at 45 ^{o}C till its completion was monitored by TLC. After the reaction was completed, it was diluted with water and extracted with ethylacetate (2×20 ml). Organic layer was dried over sodium sulphate and purified through column chromatography to give pure **9a-9l** in 72-98 % yield.

Compound 9a:



Yellowish gummy liquid:

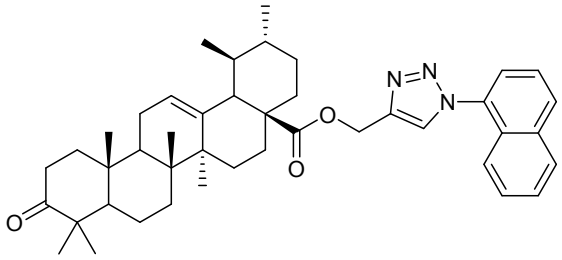
^{1}HNMR (400MHz, $CDCl_3$, δ): 8.03 (1H, s), 7.73 (2H, t, J = 8.5 Hz),7.53 (2H, t, J = 15.5 Hz), 7.44 (1H, t, J = 15.5), 5.25 (3H, m), 2.48 (1H, m), 2.31 (3H, m), 1.87 (2H, m), 1.60 (3H, m), 1.50 (3H, s), 1.44 (6H, s), 1.38 (3H, s), 1.20 (3H, s), 0.53 (3H, s).

^{13} C NMR (100 MHz, $CDCl_3$, δ): 217.8, 177.5, 14.7, 138.0, 136.9, 129.7, 128.8, 125.4, 122.4, 120.4, 77.2, 77.0, 76.7, 57.2, 52.9, 48.2, 47.3, 46.6, 39.2, 39.1, 38.8, 36.6, 36.6, 34.1, 29.7, 26.6, 23.3, 23.3, 21.4, 21.1, 19.5, 16.9, 16.7, 15.1.

IR (KBr): 2926, 2858, 1707, 1697, 1455 cm^{-1}

Mass (ESI-MS): 633.42$[M+Na]^{+}$

Compound 9b:



White solid; mp: 285 ºC

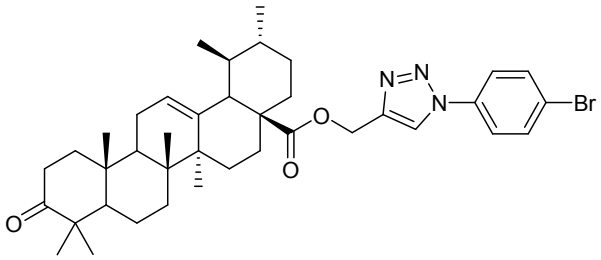
^{1}HNMR (400 MHz, $CDCl_3$, δ): 8.03 (1H, t, J = 8 Hz), 7.97 (2H, t, J = 8 Hz), 7.66 (2H, d, J = 8 Hz), 7.57 (3H, m), 5.42 (3H, m), 2.50 (1H, m), 2.34 (1H, m), 2.01 (1H, t, J = 4 Hz), 1.81 (3H, m), 1.74 (3H, m), 1.54 (3H, s), 1.03 (3H, s), 0.97 (3H, s), 0.93 (3H, s), 0.89 (3H, s), 0.86 (3H, s), 0.64 (3H, s).

^{13} C NMR (100 MHz, $CDCl_3$,δ): 217.7, 177.6, 143.2, 138.2, 134.2, 133.6, 130.5, 128.4, 127.9, 127.1, 126.6, 125.5, 124.9, 123.4, 122.4, 77.3, 55.2, 52.9, 48.3, 47.3, 46.7, 42.2, 39.5, 39.3, 39.13, 38.83, 36.70, 36.6, 34, 32.5, 26.6, 23.4, 23.4, 21.5, 21.1, 19.6, 17.1, 17.0, 15.1.

IR (KBr): 2919, 2850,1709, 1692, 1542,1449, 1383 cm^{-1}

Mass (ESI-MS): 684.24 $[M+Na]^{+}$

Compound 9c:



White papery solid; mp:279 °C

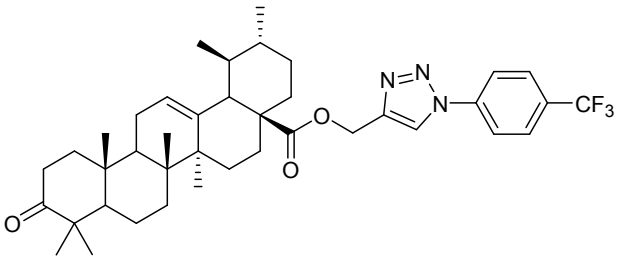
^{1}HNMR (400MHz,$CDCl_3$, δ): 8.08 (1H, s), 7.72 (4H, dd, J = 8, 8 Hz), 5.31 (3H, m), 2.50 (1H, m), 2.59 (1H, m), 2.43 (1H, m), 2.33 (1H, d, J = 12 Hz), 2.07 (2 H, m), 1.95 (3H, m), 1.35 (3H, s), 1.13 (3H, s), 1.04 (3H, s), 1.0 (3H, s), 0.99 (3H, s), 0.93 (3H, s), 0.61 (3H, s).

^{13} C NMR (100 MHz, $CDCl_3$, δ): 216.6, 176.5, 143.0, 137.1, 134.9, 131.9, 124.5, 121.5, 121.3, 120.8, 56.1, 54.2, 52.0, 47.2, 46.3, 45.7, 41.2, 38.5, 38.1, 37.8, 35.6, 33.1, 31.5, 25.6, 23.1, 22.4, 22.3, 20.4, 20.1, 16.0, 15.8, 14.1.

IR (KBr):

2919, 2854, 1721, 1705, 1588, 1490, 1457, 1394 cm^{-1} Mass (ESI-MS): 712.94$[M+Na]^{+}$

Compound 9d:



Light yellow solid; mp: 282 °C

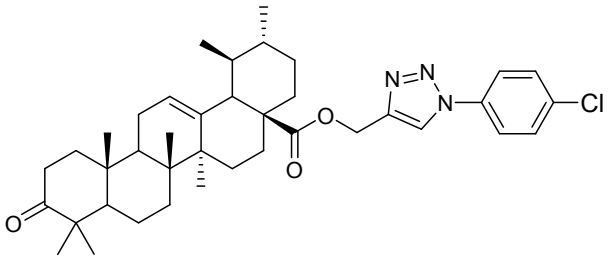
^{1}HNMR(400MHz, $CDCl_3$, δ): 8.11 (1H, s), 7.90 (2H, d, J = 4 Hz), 7.81 (2H, d, J = 8 Hz), 5.29 (3H, m), 2.51 (1H, m), 2.35 (1H, m), 2.25 (2H, d, J = 8), 1.28 (3H, m), 1.05 (3H, s), 0.99 (3H, s), 0.94 (3H, s), 0.88 (3H, s), 0.86 (3H, s), 0.84 (3H, s), 0.51 (3H, s).

^{13}C NMR (100 MHz, $CDCl_3$, δ): 217.7, 177.6, 144.2, 139.3, 138.0, 127.1, 127.1, 125.5,122.4, 120.3, 55.2, 53.0, 48.2, 47.3, 46.6, 42.1, 39.4, 39.2, 39.1, 38.8, 36.6, 34.1, 26.5, 23.4, 23.3, 21.4, 21.1, 17.0, 16.7, 15.0.

IR (KBr): 2926, 2862, 1731, 1701, 1610, 1512, 1455, 1389 cm^{-1}

Mass (ESI-MS): 701.94$[M+Na]^+$

Compound 9e:



White gummy liquid :

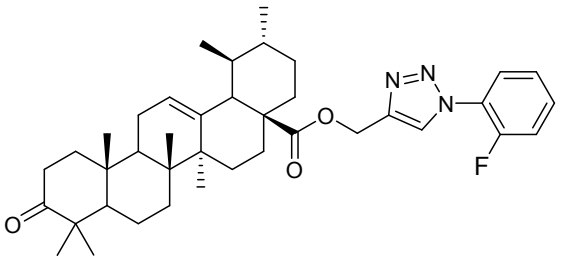
^{1}HNMR (400MHz, $CDCl_3$, δ): 8.01 (1H, s), 7.68 (2H, d, J = 4 Hz), 7.51 (2H, d, J = 4 Hz), 5.24 (3H, m), 2.61 (1H, m), 2.42 (2H, m), 2.12 (2H, m), 1.90 (3H, m), 1.3 (3H, s), 1.15 (6H, s), 1.05 (3H, s), 0.90 (3H, s), 0.85 (3H, s), 0.52 (3H, s).

^{13} C NMR (100 MHz, $CDCl_3$, δ): 217.8, 177.6, 144.0, 138.0, 135.4, 134.7, 129.9, 125.5, 122.4, 121.6, 57.1, 55.2, 52.9, 48.2, 47.4, 46.7, 42.1, 39.4, 39.3, 39.1, 38.8, 36.6, 34.1, 32.4, 26.5, 24.1, 23.4, 23.3, 21.4, 21.1,19.5, 17.0, 16.7, 15.1.

IR (KBr): 2924, 2853, 1716, 1701, 1604, 1496, 1446, 1392 cm^{-1}

Mass (ESI-MS): 668.52$[M+Na]^+$

Compound 9f:



White solid, mp: 279°C

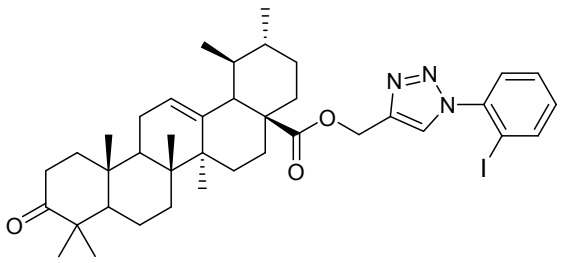
^{1}HNMR (400MHz, $CDCl_3$, δ): 8.12 (1H, s), 7.98 (1H, t, *J* = 8 Hz), 7.46 (1H, m), 7.32 (2H, m), 5.27 (3H, m), 2.51 (1H, m), 2.37 (1H, m), 2.26 (1H, d, *J* = 8 Hz), 2.01 (2H, m), 1.86 (3H, m), 1.70 (4H, m), 1.62 (3H, s), 1.30 (3H, s), 1.08 (3H, s), 1.02 (3H, s), 0.94 (3H, s), 0.86 (3H, s), 0.58 (3H, s).

^{13}C NMR (100 MHz, $CDCl_3$, δ): 217.9, 177.4, 143.5, 138.1, 130.3, 130.2, 125.5, 125.3, 125.2, 124.8, 117.2, 117.0, 57.2, 55.1, 52.9, 48.2, 47.3, 46.7, 42.1, 39.4, 39.3, 39.1, 38.8, 36.6, 34.1, 32.5, 29.7, 26.6, 24.1, 23.4, 21.4, 21.1, 19.5, 17.0, 16.7, 15.1.

IR (KBr): 2926, 2859, 1716, 1695, 1586, 1522, 1481, 1390, 1342, 1309 cm^{-1}

Mass (ESI-MS): 652.12 $[M+Na]^+$

Compound 9g:



Yellow paste like:

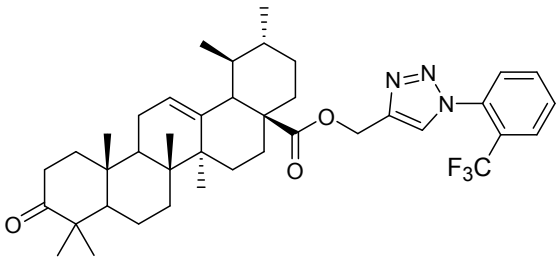
^{1}HNMR (400MHz, $CDCl_3$, δ): 8.01 (1H, d, J = 4 Hz), 7.91 (1H, s), 7.51 (1H, t, J = 12 Hz), 7.45 (1H, d, J = 4 Hz), 7.26 (1H, m), 5.26 (3H, m), 2.51 (1H, m), 2.38 (1H, m), 2.26 (1H, d, J = 8 Hz), 2.01 (2H, m), 1.90 (3H, m), 1.70 (4H, m), 1.28 (3H, s), 1.12 (3H, s), 1.05 (3H, s), 1.02 (3H, s), 0.95 (3H, s), 0.86 (3H, s), 0.74 (3H, s).

^{13}C NMR (100 MHz, $CDCl_3$, δ): 217.8, 177.4, 143.0, 140.4, 139.9, 138.2, 131.5, 129.2, 127.8, 125.9, 125.4, 93.6, 57.3, 55.2, 52.9, 48.2, 47.4, 46.7, 42.2, 39.5, 39.3, 39.1, 38.8, 36.7, 36.6, 34.1, 32.5, 30.6, 29.7, 28.0, 26.6, 24.1, 23.5, 23.4, 21.5, 21.1, 19.6, 17.1, 17.0, 15.3.

IR (KBr): 2929, 2842, 1711, 1698, 1581, 1529, 1462, 1404, 1379, 1322 cm^{-1}

Mass (ESI-MS): 759.94 $[M+Na]^+$

Compound 9h:



Grey white solid, mp: 283 ^{o}C

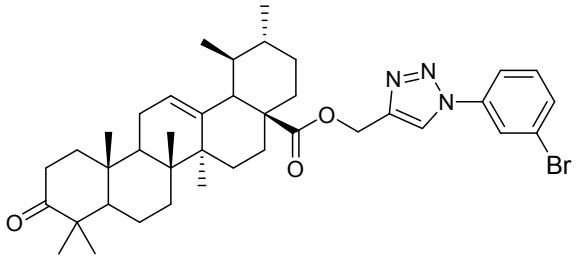
^{1}HNMR(400MHz, $CDCl_3$, δ): 7.88 (1H, s), 7.87 (1H, s), 7.72 (2H, m), 7.56 (1H, m), 5.26 (3H, m), 4.09 (1H, d, J = 4 Hz), 2.52 (1H, m), 2.37 (1H, m), 2.26 (1H, d, J = 12 Hz), 2.01 (2H, m), 1.90 (3H, m), 1.57 (3H, s), 1.28 (3H, s), 1.10 (3H, s), 1.05 (3H, s), 1.01 (3H, s), 0.95 (3H,s), 0.87 (3H, s), 0.74 (3H, s).

^{13}C NMR (100 MHz, $CDCl_3$, δ): 217.5, 177.4, 143.2, 138.2, 133.0, 130.5, 128.9, 127.4, 126.5, 125.4, 57.2, 52.9, 48.2, 46.7, 42.2, 39.5, 39.3, 39.1, 38.8, 36.7, 36.5, 34.2, 32.5, 30.6, 29.7, 28.0, 26.6, 24.1, 23.4, 21.5, 21.1, 19.6, 17.0, 15.2;

IR (KBr): 2926, 2854, 1728, 1701, 1611, 1512, 1456, 1389 cm^{-1}

Mass (ESI-MS): 701.94$[M+Na]^+$

Compound 9i:



Grey white solid, mp :280 ^{o}C

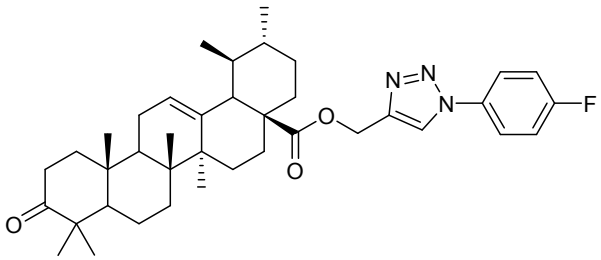
^{1}HNMR (400MHz, $CDCl_3$, δ): 8.01 (1H, s), 7.92 (1H, s),7.68 (1H, d, *J* = 8 Hz), 7.58 (1H, m, *J* = 8 Hz), 7.42 (1H, t, *J* = 12 Hz), 5.27 (3H, m), 2.69 (1H, m), 2.40 (1H, m), 2.28 (1H, d, *J* = 12 Hz), 2.16 (3H, m), 2.01 (2H, m), 1.69 (3H, m), 1.25 (3H, s), 1.05 (3H, s), 0.99 (3H, s), 0.87 (3H,s), 1.01 (3H, s), 0.95 (3H, s), 0.87 (3H, s).

^{13}C NMR (100 MHz, $CDCl_3$, δ): 217.8, 177.6, 144.0, 138.0, 137.8, 131.8, 131.1, 130.9, 128.8, 125.5, 123.6, 123.3, 122.6, 118.9, 71.8, 55.1, 53.0, 48.2, 47.3, 46.6, 42.1, 39.4, 39.1, 38.8, 36.6, 34.1, 30.9, 29.7, 26.6, 23.3, 21.4, 21.1, 17.0, 16.7, 15.1;

IR (KBr): 2922, 2851, 1728, 1705, 1592, 1493, 1456 cm^{-1}

Mass (ESI-MS): 712.94 $[M+Na]^{+}$

Compound 9j:



White paste:

^{1}HNMR (400MHz, $CDCl_3$, δ): 8.09 (1H, s), 7.90 (2H, d, *J* = 8 Hz), 7.80 (2H, d, *J* = 8 Hz), 5.27(3H, m), 2.50 (1H, m), 2.36 (1H, m), 2.25 (1H, d, *J* = 11.2 Hz), 2.16 (1H, s), 1.77 (2H, m), 1.25 (3H, s), 1.06 (3H, s), 1.01 (3H, s), 0.93 (3H, s), 0.87 (3H, s), 0.85 (3H, s), 0.54 (3H, s).

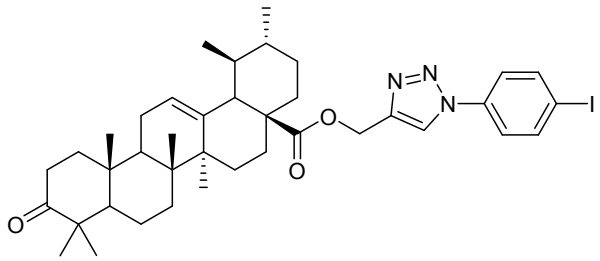
^{13}C NMR (100MHz, $CDCl_3$, δ): 217.9, 177.6, 144.2, 139.3, 138.1, 138.0, 131.0, 130.7, 127.1, 127.1, 127.1, 125.5, 125.2, 124.8, 124.5, 122.4, 120.3, 57.0, 55.2, 53.0, 48.2, 46.6, 42.1, 39.4, 39.1, 38.8, 36.6, 36.6, 34.1, 29.7, 26.5, 24.1, 23.4, 23.3, 21.4, 21.1, 17.0, 16.8, 15.0;

IR (KBr): 2928, 2850, 1713, 1705, 1622, 1539, 1435, 1382, 1345, 1310

cm^{-1}

Mass (ESI-MS): 652.12 $[M+Na]^+$

Compound 9k:



Yellow paste like :

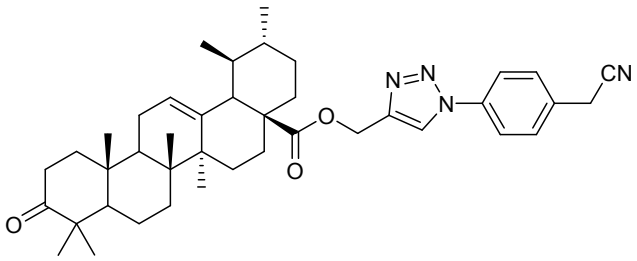
^{1}H NMR (400MHz, $CDCl_3$, δ): 8.01(1H, s), 7.68 (2H, d, J = 8.8 Hz), 7.50 (2H, d, J = 8.8 Hz), 7.45 (1H, d, J = 4 Hz), 7.26 (1H, m), 5.26 (3H, m), 2.37 (1H, m), 2.25 (1H, m), 2.23 (1H, d, J = 8 Hz), 2.16 (2H, t, J = 4.2 Hz), 1.89 (2H, m), 1.85 (3H, m), 1.26 (3H, s), 1.06 (3H, s), 1.01 (3H, s), 0.93 (3H, s), 0.88 (3H, s), 0.84 (3H, s), 0.53 (3H, s).

^{13}C NMR (100 MHz, $CDCl_3$, δ): 217.6, 177.6, 144.0, 138.1, 135.4, 134.7, 129.9, 125.5, 122.3, 121.6, 57.1, 55.2, 53.4, 53.0, 48.2, 47.4, 46.7, 42.2, 39.5, 39.3, 39.1, 38.8, 36.6, 34.1, 32.5, 31.9, 30.9, 30.6, 29.7, 29.4, 28.0, 26.6, 24.2, 23.4, 23.3, 22.7;

IR (KBr): 2922, 2850, 1705, 1504, 1459, 1375, 1329cm^{-1}

Mass (ESI-MS): 759.96$[M+Na]^+$

Compound 9l:



Yellowish White solid, mp :284 ^oC

^{1}H NMR (400MHz, $CDCl_3$, δ): 8.04 (1H, s), 7.75 (2H, d, J = 4.2 Hz),7.48 (2H, d, *J* = 4 Hz), 5.26 (3H, m), 3.66 (2H, s), 2.47 (2H, m), 2.26 (2H, m), 1.97 (3H, m), 1.40 (3H, s), 1.21 (3H, s), 1.05 (3H, s), 0.90 (3H, s), 0.85 (3H, s), 0.52 (3H, s).

^{13}C NMR (100 MHz, $CDCl_3$, δ): 217.6, 177.5, 144.1, 138.3, 135.7, 135.0, 129.7, 124.4, 122.9, 122.2, 57.3, 54.2, 52.5, 48.7, 46.1, 45.7, 43.7, 41.3, 38.7, 38.1, 38.0, 36.8, 35.3, 33.6, 29.7, 26.4, 24.6, 23.8, 22.5, 21.6, 20.9, 17.8, 16.2, 15.8.

IR (KBr): 2926, 2848, 1709, 1697,1517, 1462,1424, 1386, 1324 cm^{-1}

Mass (ESI-MS): 673.44$[M+Na]^+$

3.5. Anticancer activity:

3.5.1. Human Cancer cell lines and culture:

The optimum density of seeded cell suspension was introduced to each well of 96-well plates (Iwaki) and exposed to a range of isolated and modified constituent concentrations. Cells were cultured in (Indian Institute of Integrative Medicine, CSIR, Jammu, India) humidified atmosphere at 37 ^{o}C in 5% CO_2. In the cultured RPMI-1640 medium, known cytotoxic agents, Paclitaxel and Mitomycin-C (Sigma–Aldrich, Madrid, Spain) were used as positive controls. The cells were incubated with sample for 48 hours and fixed in cold ice TCA for 1 h at 4 ^{o}C. Plates were washed with distilled water and allowed to dry in the air. Sulpharhodamine-B (SRB) solution (0.4%) was added to each well of 96-well plates and allowed to stain at room temperature for 30-min. The unbound SRB solution was removed by washing the plates quickly with 1% (v/v) acetic acid. The bound SRB dye was solubilised by adding 100 µl of 10 mM unbuffered Tris base (PH = 10.5) to each well and shaken for 5 min on shaker platform. The plates were read in a 96-well plate reader at 540 nm.

3.5.2. Cytotoxicity assay:

Sulpharhodamine-B assay was performed against four human cancer cell lines namely THP-1 (leukemia), A-549 (lung), HEP-2 (liver) and Caco-2 (colon), which revealed increase in growth of inhibition during 48 h incubation at all concentrations of 10, 20, 40, 60, 80 and 100 µg/ml of isolated compounds. DMSO control was set up separately to cancel out the cell death occurred by DMSO, which was used as a solvent for dissolving samples homogeneously. Cytotoxicity is expressed as concentration of drug inhibiting cell growth by 50%

(IC_{50}). The results showed that the inhibition of different human cancer cell lines of varying tissue origin with 10, 20, 40, 60, 80 and 100 µg/ml of sample concentration imparted significant cellular cytotoxic effects on all the cell lines particularly on leukemia and colon cancer cells.

In case of synthetically modified compounds, all were assayed for *in vitro*cytotoxic potential against cancer cell lines including A-549 (lung), MCF-7(breast), HCT- 116 (colon), THP-1 (leukemia) and a normal human epithelial cell line Fr-2 using same protocol and standards. All these compounds were initially screened at 50 µmol concentration to get the percentage of cell growth inhibition and compounds which showed significant cytotoxic activity were further evaluated at different concentrations of 30, 10 and 5µmol concentration to get the IC_{50} values.

3.6. Determination of antimicrobial activity:

3.6.1. Microbial Strains and culture media:

The antibacterial activity of isolated and synthetically modified constituents was tested against a panel of seven bacterialand two fungal strains which were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH) Chandigarh, India. The bacterial and fungal strains used were *Bacillus subtilis* MTCC-441, *Staphylococcus epidermidis* MTCC-435, *Pseudomonas aeruginosa* MTCC-1688, *Staphylococcus aureus* MTCC-96*, Salm onella typhi* MTCC-531*, Shigella dyssenteriae* MTCC-369*, Klebsiella pneumoni a* MTCC-19*, Penicillium chrysogenum* MTCC-947 and *Aspergillus niger* MTCC-1344. Bacterial strains were grown on nutrient agar plates at 37 ^{o}C and maintained on nutrient agar slants, while as the fungal strains were grown on potato dextrose agar (PDA) plates at 25 ^{o}C and maintained on PDA slants. Cell suspension of microorganisms in NaCl 0.9% was adjusted at 0.5 McFarland to obtain approximately 10^6 cfu/ml.

3.6.2. Antimicrobial activity:

The antibacterial and antifungal susceptibility tests were carried out using the agar well diffusion assay with some modification. The overnight cultures of the indicator strains of bacteria and 72 hour cultures of fungi were added to 20 ml of liquid nutrient agar and potato dextrose agar (PDA), respectively. The contents of the tubes were transferred to petri plates. After 10 minutes of solid

the agar petri plates at room temperature, the punched wells on the plates were filled with 20 µl of 5 molar solution of each constituent in DMSO and pure DMSO was used as control. The incubation was carried out for 24 h at 37^{o}C for bacteria and 72 h at 28 ^{o}C for fungi. After the incubation period, the antimicrobial activity was evaluated by measuring the width of the zones of inhibition. Ampicillin and Vancomycin (10µg/disc) were used as standards for bacteria, while as fluconazole (10µg/disc) was used as standard antifungal drug.

3.6.3. Determination of minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)/minimum fungicidal concentration (MFC):

The MIC/MFC of constituents was determined by microdilution method, recommended by National Committee for Clinical Laboratory Standards (NCCLS) as described previously (Ashour et al., 2009). The isolates were dissolved in dimethyl sulphoxide (2.56 mg/ml) and added to the medium, and then diluted two fold to obtain concentrations in the range of 0.125-512 µg/ml (0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256 and 512 µg/ml). Inoculum suspension with a final concentration of $0.5x10^{6}$ cfu/ml was added to a 96-well micro plate. The MIC was defined as the lowest concentration of compound at which the microorganism does not demonstrate any visible growth after incubation at 37 ^{o}C for 24 h. The MBC/MFC was defined as the lowest concentration of constituents at which incubated microorganisms were completely killed.

3.7. Statistical analysis:

Each experiment was done in triplicate, and mean values were calculated. The data were recorded as respective means ± standard deviations.