SECTION 3

ANALYSIS OF BIOLOGICAL ACTIVITY OF THE ISOLATED ALKALOIDS
1. ANTIMICROBIAL ANALYSIS

3.1.1 Introduction

It is already discussed in the earlier sections, the importance of natural products in modern medicine. The pharmaceutical industry is largely based upon exploiting the applicability of compounds isolated from natural sources per se, by synthesizing and molding them to usable form or by mimicking their active sites in a more economical way.

Microorganisms namely bacteria, viruses, fungi and other parasites are present everywhere and are responsible for a large number of infectious diseases. The antimicrobial drugs are the greatest contribution of the 20th century to therapeutics and are one of the few curatives that are designed to inhibit or kill the infecting organism and have no or minimal effect on the recipient. When such compounds are produced by microorganisms themselves, they are called antibiotics which can suppress the growth of or kill other microorganisms even at very low concentrations. The antimicrobial agents are usually sulfonamides, diaminopyramidines, quinolones, tetracyclins, nitrofurans or nitrobenzene derivatives. They generally act by inhibiting cell wall synthesis, by causing leakage from cell membrane, inhibiting protein synthesis or interfering with intermediary metabolism. Several problems are but frequently met with on prolonged use of antimicrobial agents. They are found to produce toxic responses like such as local irritancy and systemic toxicity, hypersensitivity reactions, drug resistance, super infection and nutritional deficiencies1.

There are several examples of compounds from plant source with antimicrobial properties. Artemisinin isolated from Artemisia annua, used in
China to treat malaria is a sesquiterpene lactone prescribed in combination therapies to fight chloroquin-resistant *Plasmodium falciparum*. Berberine from *Berberis* species is used for the treatment of leishmaniasis, pristimerin from *Celastrus paniculata* is another antimalarial agent, quassinoids from *Ailanthus* species is an antiprotozoan, plumbagin from *Plumbago indica* function as an antibacterial and antifungal, allicin from *Allium sativum* also act as antifungal and ricin from *Ricinus communis* and emetine from *Cephaelis ipecacuanha* are used to treat amoebiasis.

### 3.1.2 Present Study

Six pure compounds 6-acetonylidihydrochelerythrine (ZX), skimmianine (ZO), 7,8-dehydro-1-methoxyrutaecarpine (ZY), arnottianamide (ZQ) and the new compound, 5,12-dihydro-12-oxoindolo[2,1-b]quinazoline-6-carboxylic acid (ZW) and the sodium borohydride reduction product of ZX, 6-(2-Hydroxypropyl)-dihydrochelerythrine (ZXR) were obtained as a result of the phytochemical studies carried out on *Zanthoxylum rhetsa*. The isolation procedures as well as the conversion of ZX to ZXR are described in section 2.4. All these compounds are alkaloids with distinctive structural features which may be exploited for the welfare of mankind. More research on them is hence worth. Based on this fact they were analysed for their biological activity. This chapter describes their activity against different microbial test organisms.

### 3.1.3 Experimental

The organisms used for the study are *Escherichia coli*, *Bacillus megaterium*, *Microbotryum violaceum* and *Chlorella fusca*. These organisms were chosen because they are nonpathogenic and they had in the past proved to be accurate initial test organisms for antibacterial, antifungal, and antialgal/herbicidal activities.
3.1.4 Agar Diffusion Assay for antimicrobial Activity

The agar diffusion assays were as employed by Schulz and coworkers\(^4\). The compounds were dissolved in acetone at a concentration of 2 mg/mL. Twenty-five microliters of the solutions (50 µg) was pipetted onto a sterile filter disk (Schleicher & Schuell, 9 mm), which was placed onto an appropriate agar growth medium for the respective test organism and subsequently sprayed with a suspension of the test organism. The Gram-positive bacteria *Bacillus megaterium* and the Gram-negative bacteria *Escherichia coli* were both grown on Nutrient Broth medium (NB medium), the fungus *Microbotryum violaceum* and the alga *Chlorella fusca* were grown on Maltose Peptone Yeast (MPY) medium. The composition of the NB medium is 5.0 g peptone and 3.0 g beef extract in 1L distilled water and that of MPY medium is 0.2% [wt/vol] maltose, 0.1% peptone yeast extract and 10 mM potassium phosphate buffer to get a pH 7.5. Reference substances were penicillin, nystatin, actidione, and tetracycline. Commencing at the middle of the filter disk, the radius of the zone of inhibition was measured in millimeters which can be correlated to the activity against the microorganism.

3.1.5 Result and discussion

The activity of each compound against the microorganisms under study can be concluded from their respective zones of inhibition radii which are given in Table.3.1a. It was observed that all six compounds exhibited biological activity, hampering the growth of one or the other organism.

Considering the susceptibilities of Gram-negative bacteria *Escherichia coli* and the Gram-positive bacteria *Bacillus megaterium* to the compounds, except ZO, all others were found to inhibit atleat one of them and hence are antibacterial.
Table 3.1a: Biological activities of the *Z. rhetsa* alkaloids against microbial test organisms in an agar diffusion assay.

<table>
<thead>
<tr>
<th>Metabolites/ control substances</th>
<th>Radius of Inhibition Zone in mm</th>
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<tbody>
<tr>
<td></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>ZW</td>
<td>8</td>
</tr>
<tr>
<td>ZY</td>
<td>7</td>
</tr>
<tr>
<td>ZQ</td>
<td>0</td>
</tr>
<tr>
<td>ZX</td>
<td>10</td>
</tr>
<tr>
<td>ZXR</td>
<td>10</td>
</tr>
<tr>
<td>ZO</td>
<td>0</td>
</tr>
<tr>
<td>acetone</td>
<td>0</td>
</tr>
<tr>
<td>penicillin</td>
<td>14 + 8 pi</td>
</tr>
<tr>
<td>tetracycline</td>
<td>18 + 16</td>
</tr>
<tr>
<td>nystatin</td>
<td>0</td>
</tr>
<tr>
<td>actidione</td>
<td>0</td>
</tr>
</tbody>
</table>

1pi = partial inhibition, i.e. there was some growth within the zone of inhibition.

Study of the weedicidal property in general is usually a tedious and time consuming process. But substances having antialgal property is also found to possess, to an extend weedicidal effects as well, which is an advantage. Hence compounds having weedicidal property are of high regard to agriculture and indirectly to the human welfare. In the current analysis, it is found that all the six substances isolated from *Z. rhetsa* were active against the alga, *Chlorella fusca*. This finding hence increases the practical
significance of the plant and synthetic weedicides of today are mostly very toxic. Hence the use of *Z. rhetsa* prickles or their extracts as weedicide can be promising.

*Z. rhetsa* grows in most parts of Kerala. The availability of the prickles is very easy. It would hence be worth studying its practical applications as a weedicide in the field. Also among these compounds, ZO along with the synthetic analogue ZXR inhibited the fungal test organism, *Microbotryum violaceum*. There is also increased recognition of plant extracts as antifungal agents because of their relatively safe status, their wide acceptance by consumers, and their exploitation for potential multi-purpose functional uses.

### 3.1.6 Comparison of the activity of ZX and the reduced product ZXR

This study gives the alteration in activity due to the functional group transformation brought about by the reduction of the carbonyl group to an alcoholic group. Both the compounds produced the same effect against the Gram-negative bacteria *Escherichia coli* which means the carbonyl group has no involvement in the mechanism of activity of the isolate against this bacterial strain. But on observing the radii of the inhibition zones of both of them against the Gram-positive bacteria *Bacillus megaterium*, it can be found that when the parent compound produced a clear inhibition to the growth of the bacteria, the reduced product was totally inactive. This result therefore proves the involvement of the carbonyl group in the mechanism leading to the anti bacterial activity of ZX against Gram-positive bacterial strain. It can also be seen that ZX which showed no inhibition against the fungal strain, *Microbotryum violaceum*, after reduction becomes antifungal where as the anti-algal activity is reduced due to conversion as is perceived from the reading against *Chlorella fusca*. 
2. CYTOTOXICITY ANALYSIS

3.2.1 Neoplasia

The term neoplasia means new growth or tumour. However, all new growths are not neoplasms since there is new growth of tissues and cells in the processes of embryogenesis, regeneration and the repair of organs. The proliferation and maturation of cells in normal adults is controlled. Some cells in our body multiply throughout life. (Labile cells) Some of them have limited proliferation (stable cells) and several others do not proliferate at all (permanent cells). On the other hand neoplastic cells lose control and regulation of replication and form an abnormal mass of tissue. Therefore, the term neoplasm can be satisfactorily defined as a mass of tissue formed as a result of abnormal, excessive, uncoordinated, autonomous, and purposeless proliferation of cells. The branch of science dealing with the study of neoplasms or tumours is called oncology. Not all neoplasms are malignant and when it is, it is generally called cancer. A malignant neoplasm have several phenotypic attributes such as excessive growth and local invasiveness and the ability to form metastasis. (tumour cells break loose from a primary mass, enter blood vessels or lymphatics and produce a secondary growth at a distant site) Invasion and metastasis are the major cause of cancer related morbidity and mortality.

3.2.2 Carcinogenesis

Carcinogenesis means induction of tumours. It is a multistep process at both the phenotypic and genetic levels. In many cases cancer is inherited in the germ line. There are a variety of extrinsic agents that can instigate cancer in organisms which are referred to as carcinogens and are broadly divided in to four groups.
1. Chemical carcinogens: Alkylating agents (cyclophosphamide, β-propiolactone), polycyclic aromatic hydrocarbons, naturally occurring products (aflatoxin B1, actinomycin D), arsenical compounds, metals, insecticides and fungicides.

2. Physical carcinogens: Ultraviolet and ionizing radiations.


4. Biologic carcinogens: Viral carcinogens (DNA oncogenic viruses like Human Papilloma Virus, herpes viruses, and RNA oncogenic viruses like HTLV)

3.2.3 Molecular Basis of Cancer

Considering carcinogenesis at a molecular level, the non lethal genetic damage lies at its heart, which may be hereditary or acquired by action of external agents such as chemicals, radiations and viruses. The three classes of normal growth regulatory genes; the growth promoting proto oncogenes, the growth inhibiting cancer-suppressor genes (antionco genes) and genes that regulate apoptosis (programmed cell deaths); are the principle targets of genetic damage.

Proto oncogenes are the cellular genes that promote normal growth and differentiation. They may become oncogenes by mutation. The oncogenes are the transforming genes present in tumour cells. For instance the over expression of the gene ‘sis’ causes astrocytoma, oesterosarcoma and that of erb-B1 causes squamous cell carcinomas of lung.

Cancer-suppresser genes regulate cell growth by controlling cell proliferation. The loss of these genes is a key event in many, possibly all, human tumours. An example is the mutation in Rb genes which usually regulates cell cycles resulting in retinoblastoma, osteosarcoma and carcinoma of breast, colon and lung. Also p53 gene that takes part in the regulation of
cell cycle and apoptosis in response to DNA damage is the basis of most human cancers. A little over 50% of human cancers contain mutations in this gene. The cancers arising due to the mutation in this gene is found to be mostly somatic and less commonly inherent\(^5\).

Genes that regulate prevent or induce apoptosis are also important variables in cancer equations\(^6\). A large family of genes that regulates apoptosis has been identified. An over expression of bcl-2 protects lymphocytes from apoptosis and allow them to survive for long period; thus there is steady accumulation of B-lymphocytes, resulting in lymphadenopathy and narrow infiltration.( B-cell lymphomas)\(^7\).

In addition to these three classes, a fourth category of genes, those that regulates repair of damaged DNA is also pertinent in carcinogenesis. The normal cells are susceptible to alterations in their DNA due to exposure to carcinogens or due to alterations resulting from errors that occur spontaneously during DNA replication. The DNA repair genes mend these non lethal damages in other genes including the above three growth regulating genes. A disability in DNA repair genes can therefore predispose to mutations in the genome which may initiate development of tumours\(^5\).

### 3.2.4 Anticancer drugs

The identification of agents that are pharmacologically active against human cancer has depended mainly upon screening of natural products and their analogs\(^8\). Studies have revealed that of the new chemical entities, about 74% of anticancer drugs are either natural products or natural product-related synthetic compounds or their mimetics\(^9\). Vinblastin\(^10\), podophyllotoxin\(^11\), cytochalasin B\(^12\), paclitaxel\(^13\) etoposide, irinotecan, gemcitabine, and methotrexate (Huryn et al. 2008) are some of the plant-derived compounds in cancer treatment. Dactinomycin, bleomycin and doxorubicin are anticancer
agents from microbial sources and citarabine originating from a marine source\textsuperscript{14}.

The anticancer drugs either kill cancer cells or modify their growth. They are classified as

1. Cytotoxic drugs: Drugs acting directly on cells.
   a. Alkylating agents: Produce highly reactive carbanions which transfer alkyl groups to cellular macromolecules which results in cross linking and abnormal base pairing.
   b. Antimetabolites: These are analogues related to normal components of DNA or coenzymes involved in nucleic acid synthesis. They completely inhibit utilization of normal substrate or get themselves incorporated forming dysfunctional macromolecules.
   c. Vinca alkaloids: They are mitotic inhibitors bind to the microtubular protein, tubulin, preventing its polymerization. They only act in mitotic phase.
   d. Taxanes: These act opposite to vinca alkaloids by enhancing the polymerization of tubulin producing abnormal arrays of the protein.
   e. Epidophyllotoxin: They are not mitotic inhibitor but arrests the cell in its G2 phase and cause DNA breaks by affecting DNA topoisomerase II function.
   f. Campothesin analogues: They interact with topoisomerase I and damage DNA during replication, act in S phase and arrest cell cycle in G2 phase.
   g. Antibiotics: They intercalate between DNA strands and interfere with its template function

2. Miscellaneous cytotoxic drugs: Drugs developed by random synthesis and tested for antitumour activity.
3. Hormons: They are not cytotoxic but modify the growth of hormone dependent tumors.

Formally cancers were treated with one drug at a time. Now generally, a combination of drugs is given as intermittent pulses to achieve total tumour kill, giving time in between for normal cells to recover\textsuperscript{15}. However selectivity of majority of the drugs is limited and they are one of the most toxic drugs used in medicine. The new therapies in oncology are therefore focusing more on enzyme inhibitors targeting signaling pathways and agents that are preferentially absorbed by tumor cells or influence vascularization and tissue adhesion or penetration than pure cytotoxic or cytostatic agents and in this perspective natural products can be of great value as biological tools as well as therapeutic agents\textsuperscript{16}.

3.2.5 Present study

The compounds arnottianamide(ZQ) and 6-acetonyldihydrochelerythrin (ZX) isolated from the acetone extract of the conical prickles on the stem bark of \textit{Z. rhetsa} and the reduced compound 6-(2-hydroxypropyl)-dihydrochelerythrine (ZXR) were analysed for their cytotoxicity against seven human cell lines of various origins. The difference in anticancer activity of ZX after conversion to ZXR was also compared.

3.2.6 Experimental

The cervical cancer cell line HeLa, hepato carcinoma cell line, Hep G2, the breast cancer cell lines SK-BR-3 & MDA-MB-231, colon cancer cell lines, HCT 116 & SW480 and the melanoma cell line, A375 were used for the study. All these cell lines were obtained from NCCS, Pune and were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, along with 100 units/ml penicillin, 50μg/ml streptomycin
and 1μg/ml of amphotericin-B and were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

For the cytotoxicity experiments, cells (3x10³ /well) were seeded in 0.2ml of the medium (DMEM with 10% FBS) in 96-well plates. After overnight incubation, various concentrations of the compounds (10-100μM) were added to the cells and after 72h, the percentage of viable cells in the wells was determined by MTT assay\textsuperscript{17}.

MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, is based on the ability of mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT to form dark blue formazan crystals, which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. The number of surviving cells is directly proportional to the level of the formazan product formed.

Briefly, 72h after the drug treatment, the drug containing media was removed and fresh media containing 25 μl of MTT solution (5mg/ml in PBS) and 75μl of complete medium were added to wells and incubated for 2h. At the end of incubation, MTT lysis buffer (20% sodium dodecyl sulphate in 50% dimethyl formamide) was added to the wells (100μl /well) and incubated for another 1h at 37°C. At the end of incubation, the optical densities at 570 nm were measured using a plate reader (Bio-Rad). The relative cell viability in percentage was calculated as \((A_{570} \text{ of treated samples} / A_{570} \text{ of untreated samples}) \times 100\).

3.2.7 Result and discussion

Among the three compounds, ZX is the most potent one (Fig 3.2a). It is very interesting to note that this compound is very specific to the colon cancer cell line, SW-480 and the cervical cancer cell line, HeLa while it is
ineffective to cancer cells of other tissue origin. Among the seven cancer cell lines studied, the cervical cancer cell line HeLa was sensitive to all the compounds and showed moderate IC_{50} values (The half maximal inhibitory Concentration, which is a measure of the effectiveness of a compound in inhibiting biological or biochemical function) in response to all the three compounds (Table 3.2a). The Hepato carcinoma cell line, HEPG2 was the most resistant (Fig 3.2a) among the cell lines studied and none of these drugs could induce cytotoxicity in this liver cancer cell line (IC_{50} value range, 156.25μM - 625μM). Among the colon cancer cell lines, HCT 116 was resistant to all the compounds studied, whilst ZX induced cytotoxicity in SW480 (IC_{50}-29.74μM). The reason for the differential action of these compounds in cancer cells of same tissue origin has to be studied further.

Table 3.2a  Comparison of IC_{50} values of the three compounds A, B and C

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC_{50} (μM)</th>
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<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>HeLa</td>
<td>52.07</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td>297.61</td>
</tr>
<tr>
<td>Hep G2</td>
<td>161.29</td>
</tr>
<tr>
<td>HCT 116</td>
<td>107.38</td>
</tr>
<tr>
<td>SW480</td>
<td>29.74</td>
</tr>
<tr>
<td>A375</td>
<td>500.00</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>71.00</td>
</tr>
</tbody>
</table>
Fig 3.2a  IC\textsubscript{50} values of the three compounds A, B and C
Similarly, among the breast cancer cell lines, SKBR3 was moderately sensitive only to ZQ (IC\textsubscript{50} = 68.49μM) while MDAMB 231, another breast carcinoma cell line was moderately sensitive to all the compounds studied (Fig. 3.2a) out of which compound ZQ (IC\textsubscript{50} = 61μM) produced maximum cytotoxicity. Similarly, ZQ caused moderate cytotoxicity in the melanoma and cervical cancer cell lines (IC\textsubscript{50} = 75μM). It is also noteworthy that, the melanoma cell line is highly resistant to the compounds ZX and ZQ (IC\textsubscript{50} = 500μM and 874.12μM respectively). Further studies are required to find out the mechanism behind the variation in their cytotoxicity towards cancer cells of various tissue origins.

On comparing the anticancer activity of ZX and ZXR, it can be concluded that the transformation of the carbonyl group of ZX to hydroxy group reduced the activity to a great extent, except in the case of SK-BR-3, indicating the important role of the carbonyl group in preventing cancer cell growth. This finding is therefore an important lead in designing anticancer drugs. The work presented in this section has been communicated to the Medicinal Chemistry Research Journal.
REFERENCE


