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In-Vitro cytotoxicity effect of *RUELLIA BRITONIANA* flower on cervical Hela cancer cells

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IN PARTIALFULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF

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Abstract

Cancer can be defined very broadly as disease in which there is uncontrolled multiplication and spreadwith in the body of abnormal forms of the body's own cells.

Strictly speaking ,one should use the term 'neoplasm' (mean ing a new growth) rather than the 'cancer'.Neoplasms which have only the characteristic of localized growth are classified as 'benign'.Neoplasms with the add itional characteristics of invasiveness and/or the capacity to metastasis are classified as 'malignant'.The term cancer is usually applied only to this later types of growth.The word 'tumour' though in reality meaning 'a local swelling'is also often use dinter changeably with cancer and will be soused now adays.

According to the World Health Organisation estimates, cancer, which caused an unprecedented 9.6 million deaths in 2018 or one in six, and worldwide the second leading cause of mortality. The current treatment approaches of cancer include surgical operations, radiation induced therapy, chemotherapy, immune modulation therapy, targeted approaches, hormonal treatment, stem cell transplantation and precision medicine. Medicinal plants and their derivatives are widely accepted as effective alternative cancer therapies. A large number of clinical trials documented the effects of herbal medicinal products for cancer patients in conjunction with traditional therapeutics on recovery, immune modulation and quality of life.

Ruellia is a genus of flowering plants commonly known as Ruellias or Wild Petunias which belongs to the family Acanthaceae. It contains about 250 genera and 2500 species. Most of these are shrubs, or twining vines; some are epiphytes. Only a few species are distributed in temperate regions. They are distributed in Indonesia and Malaysia, Africa, Brazil, Central America and Pakistan. Some of these are used as medicinal plants. Many species of the genus has antinociceptive, antioxidant, analgesic, antispasmolytic, antiulcer, antidiabetic and anti-inflammatory properties. The phytochemicals constituents: glycosides, alkaloids, flavonoids and triterpenoids have been found to be present in it. The genus has been traditionally claimed to be used for the treatment of flu, asthma, fever, bronchitis, high blood pressure, eczema, and diabetes. The objective of this research work was to understand the pharmacological and phytochemical aspects of this drug and to further find the gaps in research and endorse this genus as a step towards becoming a commercial drug. Hence, further work required is to

isolate and characterize the active compounds responsible for these activities in this plant and bring this genus plants to commercial health market to serve the community with their potential benefits.

Key words:phytochemical constituents, biological, *Ruellia*, Acanthaceae

INTRODUCTION

Cancer can be defined very broadly as disease in which there is uncontrolled multiplication and spread with in thebody of abnormal forms of the body's own cells.

Strictly speaking ,one should use the term 'neoplasm' (meaning a new growth) ratherthan the 'cancer'.Neoplasms which have only the characteristic of localized growth areclassified as 'benign'.Neoplasms with the additional characteristics of invasiveness and/orthe capacity to metastasis are classified as 'malignant'.The term cancer is usually appliedonly to this later types of growth.The word 'tumour' though in reality meaning 'a localswelling'is also often used interchangeably with cancer and will be so used now a days.

Characteristics of benign and malignant tumors:

Benign tumors are usually encapsulated by fibrous tissue, which makes it easy for surgical removal of the entire tumor in-tact. Under microscopic examination, the cells of a benign tumor closely resemble the normal surrounding cells. However, compared to normalcells that show a high percentage of cells in a growth-arrested, or quiescent stage in the cellcycle, benign tumor cells usually show a greater percentage of cells in the mitotic (dividing) stage of the cell cycle. In contrast, although some malignant tumors may be encapsulated inearly stages of their development, advanced malignant tumors exhibit no clear boundaries butusually show invasion into the surrounding normal tissues. Additionally, they can be clearly discerned from normal cells and benign tumor cells because in addition to being predominantly in a mitotic stage of the cell cycle, they usually have an abnormal number of chromosomes (aneuploidy).

Classification of malignant tumor types:

Though there are many kinds of cancer (i.e. lung, mammary, stomach, skin, etc.),malignant tumors are classified based on the embryonic origin of the normal cells from which they have developed .Normal cell so

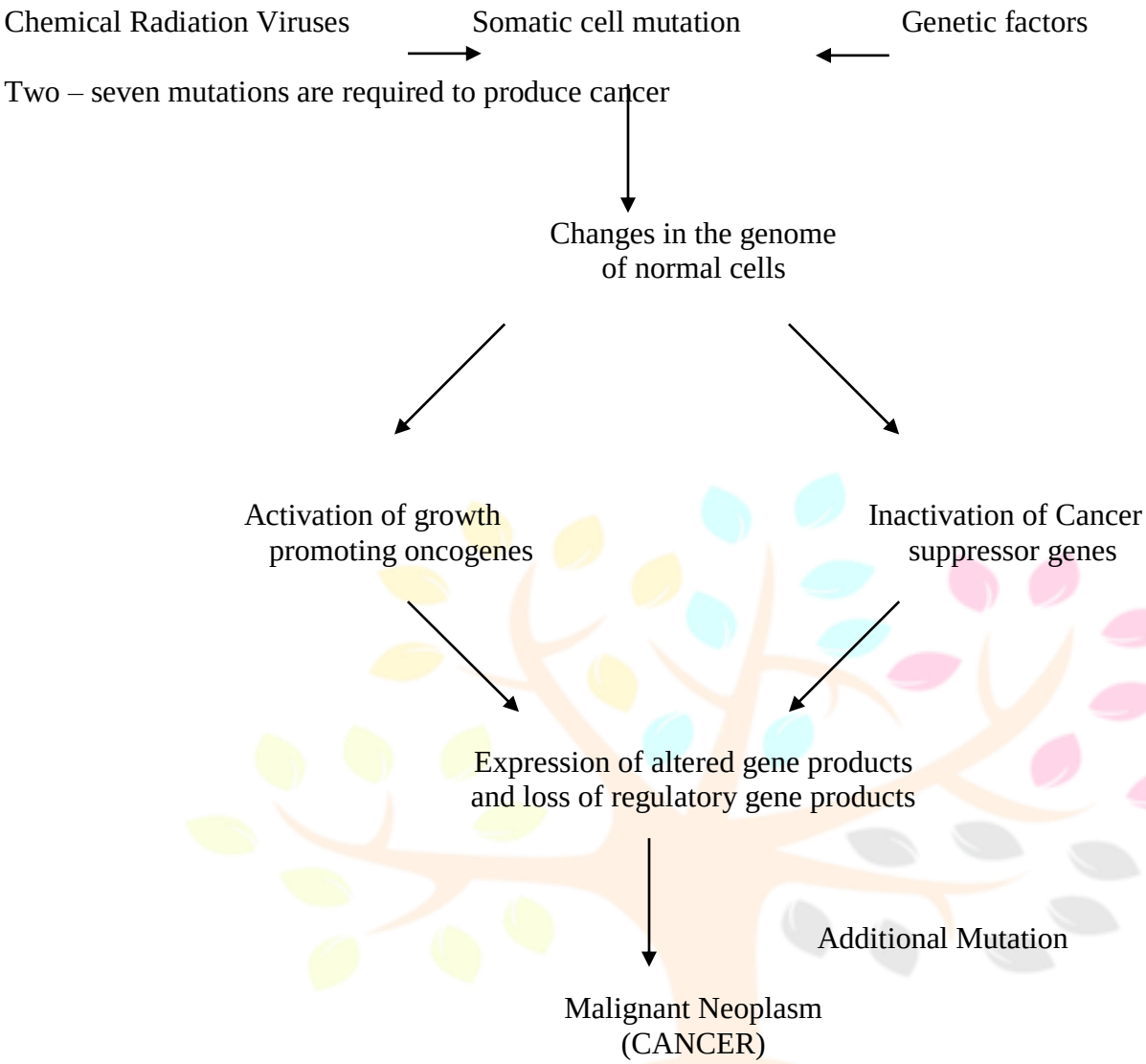
animals develop from one of three embryoniclayers :the end oderm,the ect oderm or them esoderm.

Malignant tumors derived from normal cells originating from the endoderm and the ectoderm are classified as ***carcinomas***. Malignant tumors derived from normal cells originatingin these organ systems are called ***sarcomas***. The leukemia's, cancer originating from components of the blood, are actually a subdivision of sarcomas because they grow as individual cells within the blood rather than as solid tumors.

The genesis of a cancer cell

A normal cell turns into a cancer cell because of one or more mutation in its DNA, which can be inherited or acquired. The development of cancer is a complex multistage process, involving not only more than one genetic change but also other epigenetic factors (hormonal action, co-carcinogen and tumor promoter effects, etc) that are not in themselves cancer producing but which increase the likelihood that the genetic mutation(s) will results incancer. There are two main categories of genetic change that lead to cancer

- *The activation of proto-oncogenes to oncogenes.*
- The inactivation of tumor suppressor genes.

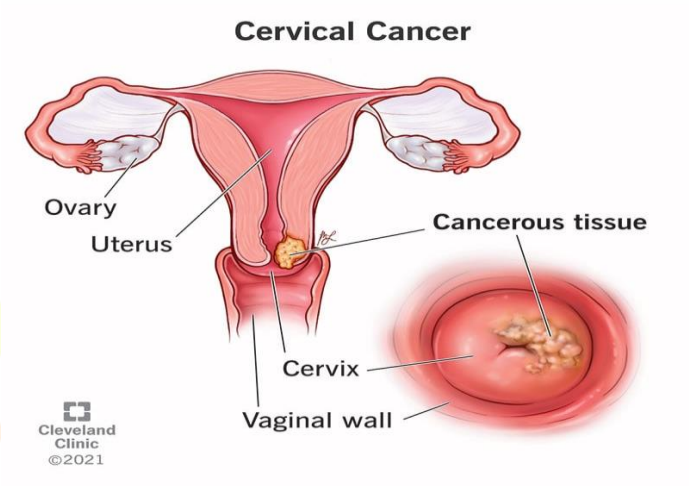


Simplified outline of the pathogenesis of cancer

Cervicalcancer:

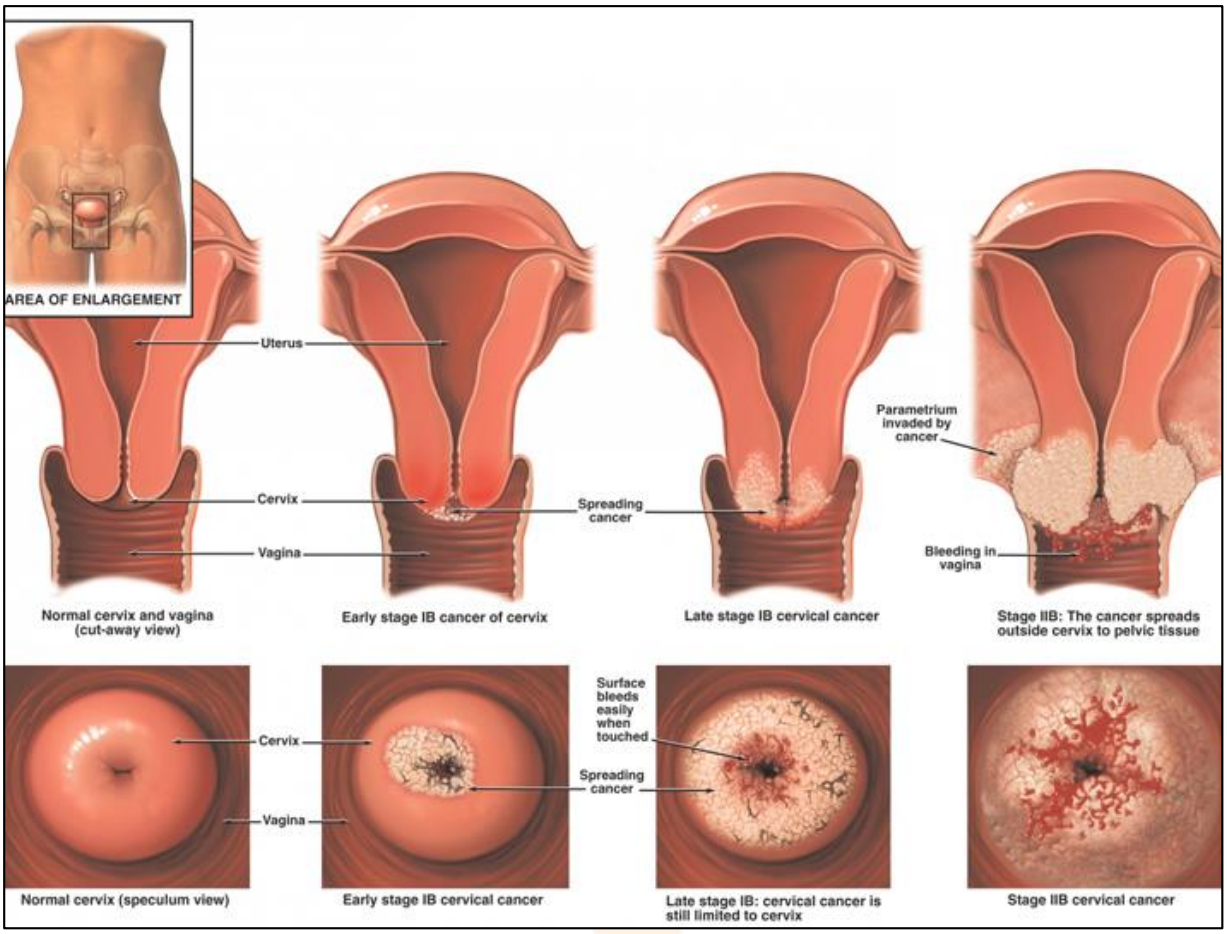
Cervical cancer is the most common cancer among women in Taiwan, with an incidence of 17.35 cases per 100,000. More than half of those affected die from this disease. In the UnitedStates alone, more than 15,700 new cases of cervical cancer are reported per year, resulting in more than 4,900 deaths. Adenocarcinoma represents about five to 20% of all cervical cancers, with the remainder being predominantly of squamous histology. More recently, the incidence of cervical adenocarcinoma has been steadily increasing worldwide, particularly in women under 35 years of age. Further more, patients with adenocarcinoma of the uterine cervix have a poor erprognosis in all stages than patients with squamous cell carcinoma. It is therefore of

great interest to employ well-developed cytogenetic and molecular techniques for further investigation and treatment of this emerging cancer. There is evidence suggesting that infection with high-risk types of human papilloma virus (HPV) is the initiating event forcervical carcinogenesis. However, as with most other cancers, environmental factors, including cigarette smoking and nutrition, appear to influence the incidence of cervical cancer. In the multistep processes of carcinogenesis underlying tumor initiation and progression, it is conceivable that additional genetic aberrations may contribute to the pathogenesis of disease.



To gain further insight into the pathogenesis of cervical adenocarcinoma, we applied comparative genomic hybridization (CGH) to analyze the genomic aberrations associated with this tumor. This method makes it possible to survey the entire genome for gains and losses of DNA sequences, without the requirement for tumor metaphases, and allows detection of any chromosomal changes that may be important in tumor development. We frequently noted, among several regions of chromosomal imbalance identified, a striking increase of copy number changes in the chromosomal arm 3q.

Stages of cervicalcancer



Staging is based on the results of a physical exam, imaging scans, and biopsies.

✓ **Stage I**: The cancer has spread from the cervix lining into the deeper tissue but is still just found in the uterus. It has not spread to other parts of the body. This stage may be divided into smaller groups to describe the cancer in more detail (see below).

✓ **Stage IA:** The cancer is diagnosed only by viewing cervical tissue or cells under a microscope. Imaging tests or evaluation of tissue samples can also be used to determine

tumor size.

❖ Stage IA1: There is a cancerous area of less than 3 millimeters (mm) in depth.

❖ Stage IA2: There is a cancerous area 3 mm to less than 5 mm in depth.

✓ **Stage IB:** In this stage, the tumor is larger but still only confined to the cervix. There isno distant spread.

❖ Stage IB1: The tumor is 5 mm or more in depth and less than 2 centimeters (cm wide.

❖ A centimeter is roughly equal to the width of a standard pen or pencil.

❖ Stage IB2: The tumor is 2 cm or more in depth and less than 4 cm wide.

❖ Stage IB3: The tumor is 4 cm or more in width.

Stage II: The cancer has spread beyond the uterus to nearby areas, such as the vagina or tissuenear the cervix, but it is still inside the pelvic area. It has not spread to other parts of the body.This stage may be divided into smaller groups to describe the cancer in more detail (see below).**Stage IIA**: The tumor is limited to the upper two-thirds of the vagina. It has not spread to thetissue next to the cervix, which is called the parametrial area.

❖ Stage IIA1: The tumor is less than 4 cm wide.

❖ Stage IIA2: The tumor is 4 cm or more in width.

Stage IIB: The tumor has spread to the parametrial area. The tumor does not reach the pelvicwall.

Stage III: The tumor involves the lower third of the vagina and/or: has spread to the pelvic wall; causes swelling of the kidney, called hydronephrosis; stops a kidney from functioning;and/or involves regional lymph nodes. Lymph nodes are small, bean-shaped organs that helpfight infection. There is no distant spread.

✓ Stage IIIA: The tumor involves the lower third of the vagina, but it has not grown into the pelvic wall.

✓ Stage IIIB: The tumor has grown into the pelvic wall and/or affects a kidney.

✓ Stage IIIC: The tumor involves regional lymph nodes. This can be detected usingimaging tests or pathology. Adding a lowercase "r" indicates imaging tests were used to confirm lymph node involvement. A lowercase "p" indicates pathology results were used todetermine the stage.

❖ **Stage IIIC1:** The cancer has spread to lymph nodes in the pelvis.

❖ **Stage IIIC2:** The cancer has spread to para-aortic lymph nodes. These lymph nodes are found in the abdomen near the base of the spine and near the aorta, a major artery that runs from the heart to the

abdomen.

Stage IVA: The cancer has spread to the bladder or rectum, but it has not spread to other partsof the body.

Stage IVB: The cancer has spread to other parts of the body.

Prevalence of cancer

All around the world, the total number of estimated cases by 2018 were about 18 million cases and out of which men (9.5 million) predominated over the women (8.5 million) population.

The most common cancers globally are listed in the table below.

The most common cases were identified as Lung and breast cancers, each contributing 12.3% of the to the cumulative cases diagnosed in 2018.

The third was identified as Colorectal cancer with 1.8 million new cases in 2018.[1]

Prevalence of cancer across the globe

Rank	Cancer	New cases diagnosed in 2018	% of all cancers (excl. non-melanoma skin cancer)
	All cancers*	17,036,901	
1.	Lung	2,093,876	12.3
2.	Breast	2,088,849	12.3
3.	Colorectal**	1,800,977	10.6
4.	Prostate	1,276,106	7.5
5.	Stomach	1,033,701	6.1
6.	Liver	841,080	5.0
7.	Oesophagus	572,034	3.4
8.	Cervix uteri	569,847	3.3
9.	Thyroid	567,233	3.3
10.	Bladder	549,393	3.2
11.	Non-Hodgkin lymphoma	509,590	3.0
12.	Pancreas	458,918	2.7
13.	Leukaemia	437,033	2.6
14.	Kidney	403,262	2.4
15.	Corpus uteri	382,069	2.2
16.	Brain, central nervous system	296,851	1.7
17.	Ovary	295,414	1.7
18.	Melanoma of skin	287,723	1.7
19.	Gallbladder	219,420	1.3
20.	Larynx	177,422	1.0
21.	Multiple myeloma	159,985	0.9
22.	Nasopharynx	129,079	0.8
23.	Oropharynx	92,887	0.5
24.	Hypopharynx	80,608	0.5
25.	Hodgkin lymphoma	79,990	0.5
26.	Testis	71,105	0.4

Existing Treatment approaches ofcancer:

Cancer treatment approaches may be classified as surgery, radiotherapy, chemotherapy and hormonal therapy.

In addition, several other novel therapies have also emerged namely immunological therapy and targeted drugdelivery.[2]

Hurdles in cancertreatments

Difficulty in targeting cancer stem cells(CSCs).

Previous studies have shown that Cancer cells arise from a cell possessing the characteristics of a stem cell. Most evidences relate to the major cause of cancerous cells which arise from an individual cell with characteristics of stem cells. Normally, in cancer therapies, it is assumed somatic cells possess malignant potential and due to the absence of specificity, these treatments have proven inefficient in terms of long-term treatment. Drugs which are target specific fail to eliminate cancer stem cells which result in tumour recurrence. Hence in order to overcome this, it is essential to understand the prognosis of particular cancertypes.[3]

The immunity against anti-cancer drugs due toresistance

In general, stem cells follow a continuous process of auto renewal and multiplication and possess the unique capability of protection from xenobiotic agents. Current research suggests that they comprise of a very small population of tumour cells. Hence the rise of targeting CSC using agents which are toxic to rapid dividing cells. Since they are not widely involved in cell division, they are not affected by such chemotherapeutic agents.[4]

Absence of cancer epigenetic profiling and specificity of currentepi-drugs

Existing research is focussed on identifying the pattern of genetic aberrations which are a direct result of mutational or chromosomal aberrations. However only a minor of these have been associated with cancer which does not explain the vast genetic deviation which results in malignant phenotype of cancer. It is an immediate necessity to develop an epigenomic drug. Hypermethylation, methylation, histone modifications are the major epigenetic alterations associated with cancer and the use of DNA methyl transferase inhibitors inversely activate the silenced oncogene. A better understanding of the human chromosomes on epigenetic patterns for cancer cells is essential. It may be essential for developing novel drugs in the early diagnosis ofcancer.[5]

Complications associated with the diagnosis of cancer make it hard totreat

The fact that cancer is non-specific, proves several difficulties in diagnosis. Hence identification of the early signs and symptoms will aid in early clinical treatment.

Difficulties associated with diagnosis of specific cancertypes:

Oesophagealcancer

One of the more severe forms of cancer, and relatively difficult to treat is oesophageal cancer. If undetected, may result in metastasis into stomach, lungs, liver and lymph nodes. This type of tumour is incurable and treatment only works by increasing the life span.[6]

Prostate cancer

Older males aged above 50 are most susceptible to Prostate cancer. Since it is difficult to identify at early stages, it metastasizes to different parts of the body. Prostate specific antigen (PSA) may be considered as a biomarker for the early detection, but the patient doesn't show any form of symptoms during the preliminary stages. If it continues being undetected, it may undergo metastasis to various other body regions, in particular bones and lymph.[7]

Pancreatic cancer

This form of cancer is not very evident at first as patients do not exhibit the symptoms, and even during the final stages, patients mostly exhibit symptoms referred to as non-specific. The symptoms are not uniform and depends on the cancerlocation.[8]

Lack of effectivebiomarkers:

Biomarkers are essential in diagnosing cancer and the decreased availability of effective biomarkers is a limitation in cancer research. Once biomarkers are identified, the therapeutic efficacy of chemotherapeutic agents may be tested and validated. A novel concept of clinical proteomics has provided sufficient promise in identifying biomarkers to aid early cancer detection, but due to certain hurdles, more research and standardization of approach is required.[9]

Difficulties associated with existing treatments

Currently, the drugs used in cancer therapy have shown to affect not just cancerous cells, but also normal ones. Hence a constant administration of these agents will eventually lead to more drastic consequences and even lead to patient death. Radiotherapy has also shown similar effects due tonon-specificity.[9]

Metastasis and Cancer

Another problem in cancer treatment is cancer metastasis and due to the non-symptomatic nature, along with difficulty in diagnosis results in the spread to cancer to other body parts. Initial cancer site is the primary site and the region it spreads to is referred to as the secondary site. In order to diversify, the primary sites possess the ability to take over other sites.

Cancer metastasis may be categorized into three types:

- Localspread
- Bloodcirculation
- Lymphaticsystem

Hence therapeutic approaches should not only look to eliminate the primary form of cancer but also target the secondary sites. Due to the difficulty in diagnosis, these metastatic events may not always be detected.

Certain difficulties associated with the cancer cells are the removal of each one of them. Surgery in this case may turn out ineffective in removing each of the cell and the treatments associated with cancer are related to certain toxic effects. The presence of even a few cancer cells may result in disease resurgence.

Anti-cancer agents have already been identified in lethal forms of cancer namely Hodgkin's lymphoma, testicular cancer, choriocarcinoma, and some leukaemia's. But the question lies as to what chances are there to developing better cures to most of the cancerforms.[9]

Toxicities and adverse effects associated withchemotherapy

Paclitaxel

Initial member of taxanes family to be utilized as a cancer chemotherapeutic agent. These agents act by by arresting mitosis through microtubule stabilization, resulting in cellular apoptosis. However, significant toxicities, such as myelosuppression and peripheral neuropathy, limit the effectiveness of paclitaxel-based treatment regimens.[10]

Doxorubicin

There are two possible mechanisms by which this drug actsInsertion into DNA (deoxyribonucleic acid) and dysregulation of DNA repair mediated by topoisomerase-II

Free radical production which damages cell components, DNA and proteins. Doxorubicin administration has been found to be linked to cardiotoxicity.[11]

Cyclophosphamide

Acts by exerting cytotoxic effects directly on tumour cells. It also results in immunosuppression in tumour, however higher doses are toxic and may also result in pericardiomyopathy.[12]

Cisplatin

It acts by combining with the DNA and intrudes the repair mechanisms causing the death of cells. Adverse effects commonly associated with cisplatin are- Nephrotoxicity, Ototoxicity, Myelosuppression and Anaphylaxis.[13]

Fluorouracil

This drug first received approval in 1962 and is referred to as one of the oldest agents prescribed. It has been prescribed for patients suffering from gastrointestinal cancers as well as breast cancer. Administration of Fluorouracil has been found to be associated with Myelosuppression, photosensitivity as well as leukopenia.[14]

Gemcitabine

A newer chemotherapeutic agent and has been found to decrease cancer growth. When administered without a combination, has been found to be effective in pancreatic cancer. Combinations have also been tested and successful in breast, ovarian and lung cancers. However, it has also been found to be related to Myelosuppression, hepatic enzyme upregulation and nausea.[15]

Risk Factors

- HPV infection: Sexually transmitted virus

HPV infections are very common.

Most men and women who are sexually active have been exposed to HPV. Over 85% of men and women have been infected with HPV at some time in their lives, but most infections clear up on their own. More than 75% of sexually active women have been exposed to HPV by age 18-22. Some types of HIV can cause changes to cells in the cervix. If these changes are found early, cervical cancer can be prevented by removing or killing the changed cells before they can become cancer cells.

- Lack of regular Cervical Smear tests:

Cervical cancer is more common among women who don't have regular smear tests. The smear test screens for abnormal cells. Removing or killing the abnormal cells usually prevents cervical cancer.

- Family History and Smoking:

Heredity/ Genetics

Smoking cigarettes increases the risk of cervical cancer.

- Weakened immune system:

The body's natural defense system: Infection or taking drugs that suppress the immune system increases the risk of cervical cancer

- Using birth control pills for a long time:

Using birth control pills for a long time (5 or more years) may slightly increase the risk of cervical cancer. However, the risk decreases quickly when women stop using birth control pills.

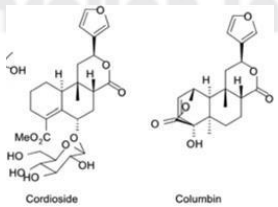
- DES (diethylstilbestrol):

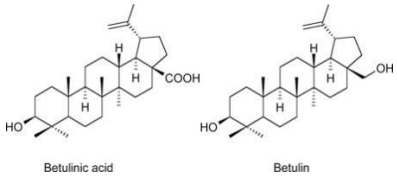
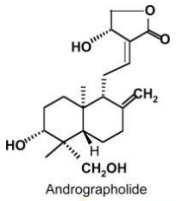
DES may increase the risk of a rare form of cervical cancer in daughters exposed to this drug before birth. DES was given to some pregnant women between 1940 and 1971.

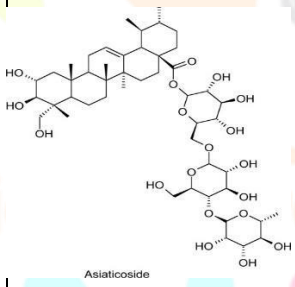
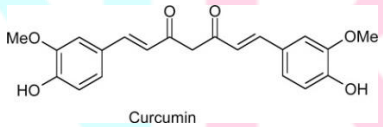
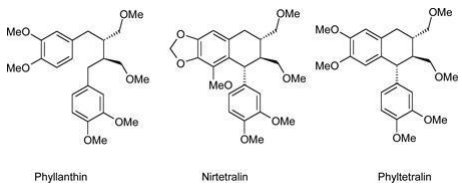
(It is no longer given to pregnant women)

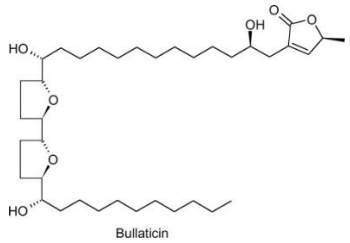
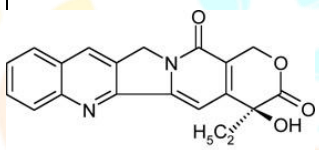
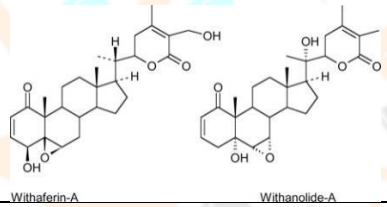
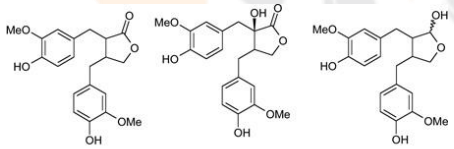
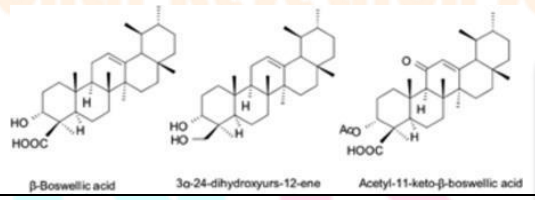
Having a HPV infection or other risk factors does not mean that a woman will develop cervical cancer. Most women who have risk factors never develop it. Women who have never been sexually active and who have not had the HIV virus can also develop cervical cancer

Medicinal plants in cancer treatment [16]

Scientific name	Anti-cancer component	Structure	Cell lines
Tinosporacordifolia	Columbin, Cordioside	 Cordioside Columbin	Hela cancer cell lines,[22] MDA- MB-231,[23] Humancolon adenocarcinoma (HCA-7),[24] C6 glioma cells.[25]

Ziziphusnummularia	Betulinic acid, Betulin		HeLa cancer cell line,[26] *p53* mutant cells, as wellas primarytumour cells.[21]
Andrographispaniculata	Andrographolide		KBhuman epidermoid cancercells, P388lymphocytic leukemiacells, MCF-7breast cancer cells and HCT-116colon cancer cells.[21]

Centellaasiatica	Asiaticoside		MCF-7 Breast cancer cell lines, mouse melanoma (B(16)F(1), human breast cancer (MDA MB-231) and rat glioma (C(6)) celllines.[21]
Curcuma longa	Curcumin		Epidermoid carcinoma cell line, colon and gastric cancersin rodents.[21]
Phyllanthus amarus	Nirtetralin, phyllanthin, phyltetralin		Dalton's lymphoma ascites (DLA) and Erlich ascites carcinoma (EAC) .[21]

Annona muricata	Bullatacin	 Bullaticin	Human solid tumour cell lines A-549 lung carcinoma, MCF-7 breast carcinoma, and HT-29 colon adenocarcinoma cell lines.[21]
Mappiafoetida	Camptothecin	 Camptothecine	HeLa cells and L-120cells.[21]
Withaniasomnifera	Withaferin A, Withanolide A.	 Withaferin-A Withanolide-A	Mouse Sarcoma-180, Ehrlich and ascites tumour models.[21]
Cedrusdeodara	Wikstromal,matairesinol and dibenzylbutyrolactol	 Metairesinol Wikstromol Dibenzylbutyrolactol	Human leukemia Molt-4 and HL- 60 cells.[21]
Boswelliaserrata	β-Boswellicacid, 3α-24-dihydroxyurs-12-ene, Acetyl-11-keto-β-boswellic acid	 β-Boswellic acid 3α-24-dihydroxyurs-12-ene Acetyl-11-keto-β-boswellic acid	MDA-MB-231 breast cancer cell line.[21]

Future perspective of cancer research

Loss of cell cycle and Genetic instability associated with cancercells

Genetic instability is a result of loss of chromosome maintenance or DNA repair. Current therapies focus on agents such as drugs and ionizing radiation. The use of Radiation results in DNA damage and machinery which maintains chromosomal integrity. These treatments are beneficial in killing cancerous cells but cause damage to the DNA of normal cells. Tumour cells are defective in the cell cycle checkpoint and cannot arrest he cell cycle, hence multiply immediately after radiation. Most of these cells die in a few days resulting from DNA damage.[17]

Cancers Can convert resistance intotherapy

Though certain defects at molecular level increase sensitivity of cancer cells to cytotoxic agen ts and others may show resistance. A majority of the cancer cell death is by DNAdamage and it takes place through apoptosis and hence cancer cells which

harbour death through cell death program may escape the effect so cytotoxic therapy. Cancer cells maydiffer in the response to the effects of radiation and drugs and it reflects in DNA repair, cell- cycle checkpoints, and apoptosis pathways.[17]

Genetic instability may be considered as both beneficial as well as harmful in anti-cancer therapy. Due to the increased mutations in cancer cells, targeting them with a single form of treatment is difficult as they evolve resistance to therapies at a drasticrate.

Sometimes these cells may also develop resistance to other drugs to which they were not exposed to (multidrug resistance). The excess production of this protein can avoid the intracellular accumulation of lipophilic drugs by pumping out of thecell.[17]

Emergence of novel therapeutic approaches

Drugs such as estrogen antagonists are used worldwide in the treatment of breast cancer. These agents do not directly target cancer cells, but prevent or delay cancer recurrence. They also enhance the survival of the

patients. However more potent therapies would involve the direct extermination of cancer cells. Several novel therapies were identified to reduce or prevent tumour progression, yet most of these were unsuccessful when it came to the clinical stage.

Some drugs may still show some potential as anti-cancer agents. Some tumour cells rely on certain proteins which they produce in excess. Hence, an effective therapy may be to block the activity of the protein provided, it does not affect the normaltissues.

A notable example for this is that about 25% of breast cancers show higher levels of Human epidermal growth factor 2 (Her2) protein, which is linked to Epidermal growth factor receptor and generally plays a role in the mammary epithelium formation. Hence, inhibiting the function of Her2 may alter the growth of the tumour and slow theprogression.

Another possible approach to this is by targeting the tumours with toxic compounds by using proteins such as Her2 which is abundant on the cell surface. Some antibodies can be filled with toxins or enzymes which cleaves prodrugs into toxic molecules. The mechanism by which it works is by enzymatic diffusion into neighboring cells increasing the chances by which it will be killed as well.[17]

Therapy to target cells lackingp53

One way of targeting tumour cells is by taking into consideration the loss of p53. For example, some viruses such as adenovirus bind and subsequently inactivate p53 of the host and hence enabling it to replicate inside the host and inactivate the defenses.

During the lytic phase, these viruses replicate inside the cells and infect the neighbors along with it. Currently an adenovirus has been created which lacks the particular gene encoding p53- blocking protein and which can replicate only within cells where the p53 sequence has been inactivated. The hypothesis suggests that if this adenovirus is injected to the tumour then the virus may replicate and kill the cancer cells which lack p53, hence causing no harm to normal cells. This strategy is currently being implemented in clinicaltrials.[17]

Inhibiting tumour growth by hindering bloodsupply

A promising approach to cancer therapy is destroying the tumour be indirectly targeting cancer cells. Since the

tumour involves formation of blood vessels, treatments which inhibit tumour growth should act on different types of cancer. The formation of new blood vessels involves several angiogenic growth factors and the mechanism of which can be potentially blocked. Angiogenesis inhibitors are now undergoing clinical trials. Some cells such as endothelial cells aid in the process of forming blood vessels and express distinct cell surface markers hence providing a path to being attacked without harm to the normal blood vessels.[17]

Targeting oncogenic proteins

Earlier it was observed that chronic myelogenous leukemia was evident in 95% of the cases and a particular chromosomal translocation is evident as an abnormality in karyotype. This is a result of breakage and re-joining of two genes- *Abl*(involved in cell signalling) and *Bcr*. The addition of *Bcr*to the N terminus results in hyperactivity and aberrant proliferation of hemopoietic precursor cells followed by inhibition of apoptosis. A large number of White blood cells (WBC) cells are released into the blood stream resulting in leukemia.

Hence the protein complex *Bcr- Abl*is a possible target for anti-cancer agents. A novel drug molecule has been discovered to inhibit the protein kinase activity known as STI-571, and this drug when administered to patients, exhibited excellent responses in returning the levels of WBC back to normal. A year of this treatment rendered 51 out of the 54 patients successful, however those patients who suffered a progression in this disease did not recover. These patients exhibited signs of recovery and finally relapsed. The success of Gleevec treatment is sufficient to provide an understanding of the lesions and improvise on therational ways of treatingit.[17]

Tailored and rational medical treatment through better understanding of mechanisms

Diagnosis is essential in order to develop an effective treatment approach. Several novel techniques have been identified to understand and make the diagnosis precise and specific.

For example, microarrays are essential to understand the messenger RNA in the tissue sample and the levels of thousands of genes can be analysed in a single sample. Each form of cancer is expressed by a specific gene profile and when compared, can be grouped into a number of distinct classes and these reflect onto the oncogenic mutations followed by the prognosis and responses to therapies.

With the current understanding of molecular mechanisms, it is possible to determine the defects associated with

DNA metabolism, Replication, recombination, repair and chromosomal maintenance, hence acquiring multiple mutations in tumour growth.

The exposure of these cells to radiation results in DNA damage due to the vulnerability of the tumours with defects. Hence by exploiting this mechanism, it is possible to attack cancer cells more effectively and tailor a therapy with much more accuracy.

It is evident that certain genetic abnormalities are shared by various disease types. The DNA sequences of some genes and the physiological functions associated with them are also known. Yet, it is essential to identify the sociology of the cells in the tissues and the processes involved in the genesis and spread of cancer by means of mutation along with natural selection.[17]

Aims:

The present study is aimed toevaluate the cytotoxicity studies of ***R. brittoniana flower*** against Human Cervical cells .

REVIEW OFLITERATURE

Botanical Name : *Ruellia simplex*

Taxonomy : Ruelliabrittoniana, Ruelliaspectabilis, Ruelliatweediana

Kingdom : Plantae

Order : Lamiales

Family : Acanthaceae

Genus : Ruellia

L Species : Ruellia simplex

Habitat : Deccan,plainsofcarnatic andCeylon.

Partsused : Leaves, flowers,roots,stemand whole plant.

Plants are being used as medicines since the beginning of human civilization; healing powers are reported to be present in plants and therefore, it is assumed that they have medicinal properties. The flora of Pakistan due to its diverse climate, soil conditions and many ecological regions is very rich in medicinal plants. According to a survey of Pakistan, about 6000 species of flowering plants have been existing, out of 6000 about 400-600 are medicinally important species[18]. From near past, it has been discovered that properties of medicinal plants are due to its active chemical compounds[19]. The discovery of drugs from medicinal plants started from the era when the isolation of drugs such as digitoxin, quinine, cocaine, and codeine had begun. The family Acanthaceae (Acanthus family) is a taxon of dicotyledonous flowering plants containing almost 250 genera and about 2500 species. Most of these are tropical herbs, shrubs, or twining vines; some are epiphytes. Only a few species distributed in temperate regions. Indonesia, Malaysia, Africa, Brazil and Central America are the main producers. Ruellia is a genus of flowering plants commonly known as Ruellias or wild petunias[18]. It is a genus of 250species, distributed in tropical and temperate regions of both the hemispheres, which is also present in Pakistan by 5 species, of which 3 are native[20]. These are popular ornamental plants. Some of them are used as medicinal plants. One of the species, *Ruelliatuberosa* has been extensively used as diuretic, anti-diabetic, antipyretic, analgesic, antioxidant[21], anti-hypertensive, gastroprotective[22] and to treat gonorrhea[20]. The phytochemical properties of *Ruelliatuberosa* has been investigated revealing the presence of alkaloids, triterpenoids, saponins, sterols and flavonoids[23]. *Ruelliaasperula*was used in bronchitis, asthma, flu, fever and uterus inflammation[24]. *Ruellia prostrate* leaf is used in the treatment of chronic rheumatism, eczema, facial

paralysis, cephalgia and hemiplegia. Leaf juice is an efficient remedy in colic of children[25]. The fresh leaf of the plant *Ruelliapatula* is pounded and then soaked in water until the solution turns black. It is decanted and the solution is applied to the ear[26]. *Ruelliahygrophila* has an antispasmodic and analgesic activity.

Taxonomy

The family Acanthaceae, Class Eudicots and order Lamiales includes 250 genera and 2500 Species[27]. In Pakistan it is represented by 18 genera and 60 specific and infra specific taxa, of which 44 are native[28]. The first palynological study was investigated few taxa[29]. However, forthcoming studies[30] carried out detailed study and recognized for the first time 11 pollen types within thefamily Acanthaceae.

Folk medicinal uses of genus*Ruellia*

The whole parts of genus Ruellia are used inbladder stones and in bronchitis. Paste of leaves is also used for skin diseases and boils. Roots are usedas anthelmintic. Syrup is used for whooping cough.Tuber powder is used for stomach ache. *Ruelliatuberosa* has been extensively used for the treatmentof emesis, analgesia, nociceptive pain, inflammation,renopathy and syphilis[31]. *Ruelliatuberosa* isused in stomach cancer.In folk medicine and Ayurvedic medicine thegenus Ruellia has been used as diuretic, anti-diabetic[32] antipyretic, analgesic, antioxidant[21] antihypertensive,gastroprotective[25] agent and wasalso used to treat gonorrhea[33].

Scientific studies of genus Ruellia Antioxidant activity

The antioxidant activity of different extracts of stem of Ruelliatuberosa were investigated by various *in-vitro* methods like 2,2-diphenyl-1-picrylhydrazyl (DPPH), free radical-scavenging assays and the hydrogen peroxide induced luminolchemiluminescence assay. The methanol extract and its four fractions of water, ethyl acetate, chloroform, and n-hexane were evaluated for antioxidant activity. The results revealed that *Ruelliatuberosa* possesses potent antioxidant activity[21]. It has been reported by other authors that the aerial parts of plant *Ruelliaprostrata* showed antioxidant potential. Different concentrations of methanolic extract and n-butanolic fraction were subjected to antioxidant assay by DPPH method, nitric oxide scavenging activity and reducing power assay. Both the n-butanolic and methanolic extract showed the antioxidant potential but the antioxidant potential of n-butanolic fraction is far higher than the methanolic extract[22].

Gastroprotective and analgesic activity

It was reported that aqueous extract of *Ruelliatuberosa* roots showed a strong and dose dependent gastroprotective activity in alcoholinduced gastric lesion of rats. The extract also had a mild erythropoietic and moderate analgesic activity. It was concluded from the data that *Ruelliatuberosa* root extracts have gastroprotective activity[23].

Anti-ulcer activity

Preliminary ethyl acetate extract of *Ruelliatuberosa* was studied for the acute oral toxicity, according to the economic cooperation and development guidelines, based on which two doses were selected, 250 mg/kg (low dose) and 500 mg/kg (high dose). Ranitidine was used as the standard drug (20 mg/kg). The ethyl acetate extract showed significant decrease in gastric volume, total acidity and free acidity. There was a significant ($p < 0.01$) increase in gastric pH only at high dose (500 mg/kg), when compared to control group. The value of ulcer index decreased in a dose dependent manner, when compared to control group[34].

***In-vitro* purgative and cholinergic activity**

Methanolic, ethyl acetate and aquous extracts of *Ruelliatuberosa* produced contractions on electrically induced contracted ileum tissue strip at the dose of 30 µg/mL.Methanolic extract of *Ruelliatuberosa* was investigated on uterus and gestation by using 350 mg/kg/day and was found to increase the number of implantation[25]. This study demonstrated that estrogenic effect may be due to flavonoid and sterol while cholinergic effect may be due to iridoid glycosides.

Antimicrobial activity

The antibacterial activities of n-hexane, dichloromethane, ethyl acetate and methanol extracts of *Ruelliatuberosa* were explored against Gram positive and Gram negative bacteria. The ethyl acetate and methanol fractions exhibited the highest rates of antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*[35].

Anticancer activity

The methanol extract of aerial part of herb *Ruelliatuberosa* possessed cytotoxicity. The minimum inhibitory concentration (IC50) for methanol extract was found to be 3.5 and 1.9 µg/mL in H460 and MDAMB231 cancer cells, respectively. Tylocrebrine was isolated from Ruelliatuberosa through bioassay directed column chromatography and elucidated its anticancer and anti-inflammatory potential[36].

Antinociceptive and anti-inflammatory activity

The ethanol extract of *Ruelliatuberosa* was evaluated for its antinociceptive and anti-inflammatory properties in experimental mice and rat models. In the hot plate test, the group that received a dose of 300 mg/kg for mice showed maximum time needed for the response against thermal stimuli and maximum possible analgesic was similar to that of diclofenac sodium. The extract at 500 and 250 mg/kg doses showed significant reduction in acetic acid-induced writhing in mice, which was similar to diclofenac sodium. The extract also demonstrated significant inhibition in serotonin and egg albumin-induced hind paw edema in rats at the doses of 100, 200 and 300 mg/kg. The anti-inflammatory properties exhibited by the extract were comparable to that of indomethacin at a dose of 5 mg/kg[37].

Cardiovascular and hypertensive activity

In pharmacological investigation, extracts of *Ruelliabrittoniana* and *Ruelliapatula* were used for cardiovascular screening .The cardiovascular experiments were carried out *in-vitro* and in vivo. In *in-vitro* studies rabbit heart was used while for in vivo, anesthetized rats were used. In in vivo experiment, extracts of *Ruelliabrittonana* and *Ruelliapatula* showed a hypertensive activity in pentothal sodium anesthetized rats[38].

Antispermatogenic activity

Aqueous extract of tuberous roots of *Ruelliatuberosa* administered orally at the dose of 50, 100 and 150 mg/kg body weight for 21 days resulted in significantly decreased sperm count in male albino rats. The results suggested that the aqueous extract of *R. tuberosa* produces antispermatogenic effect in male albino rats[39].

Antidiabetic, antihyperlipidemic and hepatoprotective activity

The methanol extract of *Ruelliatuberosa* leaves at a dose of 100 and 200 mg/kg of body weight was administered at single dose per day to diabetes-induced rats for a period of 14 days. The methanol extract of

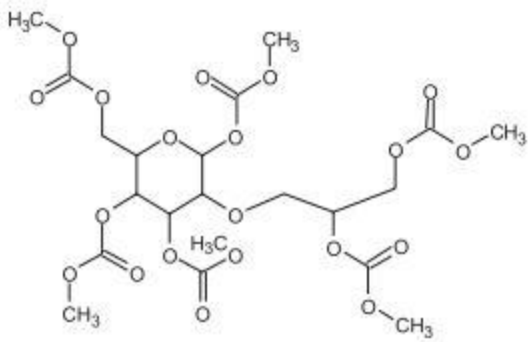
Ruelliatuberosa leaves elicited significant reductions of blood glucose ($p < 0.05$), lipid parameters except HDL-C, serum enzymes and significantly increased HDL-C at the dose of 200 mg/kg when compared with the standard drug glibenclamide (5 g/kg). From the above result, it may be concluded that methanol extract of Ruelliatuberosa leaves possesses significant antidiabetic, antihyperlipidemic and hepatoprotective effects in alloxan-induced diabetic rats[25].

Phytochemical constituents of Ruellia genus Preliminary phytochemical screening

Preliminary phytochemical screening of ethyl acetate extract of *Ruelliatuberosa* reveals the presence of saponins, tannins, and flavonoids, which may be responsible for its activity[35].

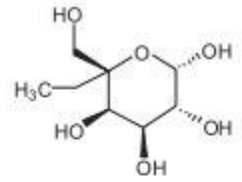
Glycosides

Extract of the whole plant of *Ruelliabrittoniana* has afforded the new glycoside 2-O-α-galactopyranosyl glycerol hexaacetate.

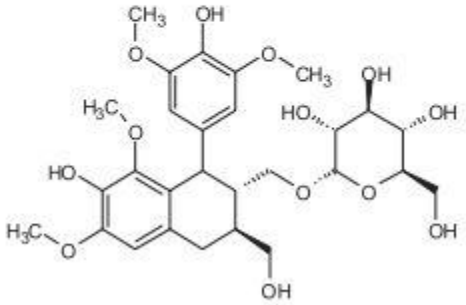


2-O-α-galactopyranosyl glycerol hexacetate

Two lignan glycosides identified as 5,5ídimethoxylariciresinol-9-α-D-glucopyranoside (reupaside) and lyoniresinol-9í-α-D-glucopyranoside with ethyl-α-D-galactopyranoside, α- and βD-glucose and β-D-fructose have been isolated from *Ruellia patula*[40,41].

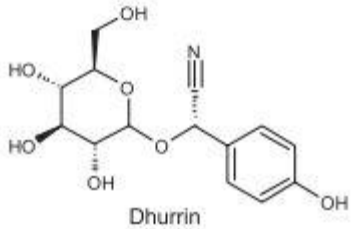


Ethyl- α-D-galactopyranose

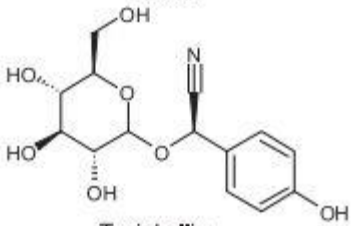


Lyoniresinol-3-β-glucopyranose

A cyanogenic glucoside was isolated from *Ruelliarosea* and showed the presence of a phydroxyphenyl moiety indicating that the compound was either taxiphyllin or the diastereomer dhurrin[42,43].



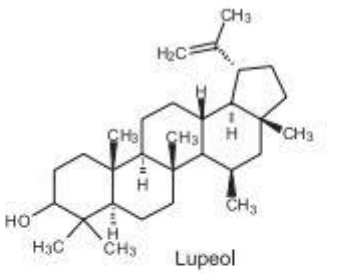
Dhurrin

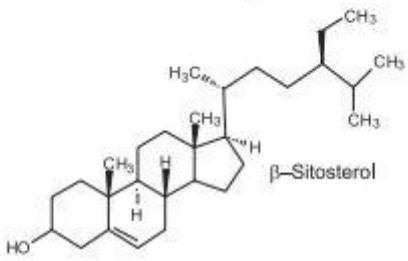


Taxiphyllin

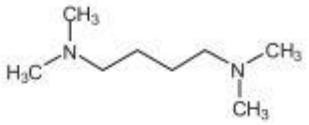
The leaves contained only traces of apigenin and luteolin, while in flowers was malvidin-3,5-diglucoside in appreciable quantity. The flowers buds contained the maximum proportion of flavonoids (3% apigenin-7-O-glucoronide and the other flavones were identified as apigenin-7-O-glucoside, apigenin7-O-rutinoside and luteolin-7-O-glucoside[43,44].

From *Ruelliapatula*, nine compounds were isolated. A new lignan glycoside 5,5-dimethoxylariciresinol-9-O-β-D-glucopyranoside, apigenin-7-Orutinoside, β-sitosterol, lupeol, α-D-glucose, β-Dglucose, and β-D-fructose[38].

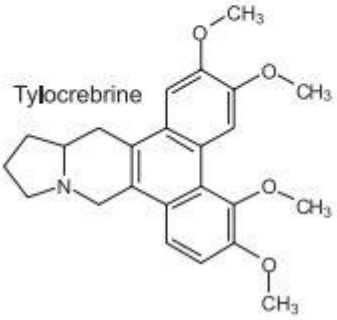




Alkaloids Tetramethylputrescine was isolated from the roots and aerial parts of *Ruellia rosea*[45]Tylocrebrine, a phenanthrene alkaloid, was reported from aerial parts of *Ruelliatuberosa* and found its anti-cancer and anti-inflammatory potential[30].



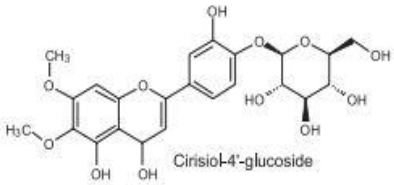
Tetramethylputrescine

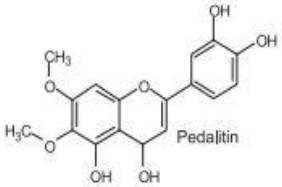


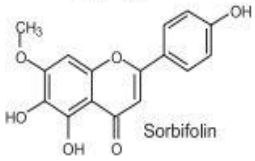
Tylocrebrine

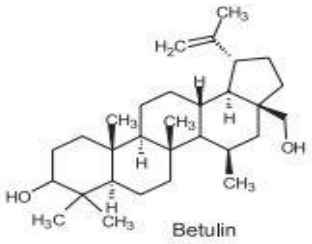
Flavonoids

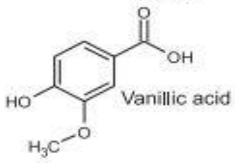
Five flavonoids: cirsimaritin, cirsimarin, cirsiliol-4í-glucoside, sorbifolin and pedalitin along with betulin, vanillic acid and indole-3-carboxaldehyde were isolated from the ethyl acetate extracts of *Ruellia tuberosa*[31]

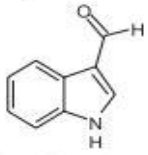












Three new flavonoid glycosides: demethoxycentaureidin 7-O-β-D-galacturonopyranoside, pectolinarigenin 7-O-α-L-rhamnopyranosyl-(1íìí,4íí)-βD-glucopyranoside and 7-O-α-L-rhamnopyranosyl- (1íìí,4íí)-β-D-glucuronopyranoside, a new megastig mane glucoside, byzantionoside B 6í-O-sulfate, and a new (Z)-hex-3-en-1-ol O-β-D-xylopyranosyl- (1íí,2í)-β-D-glucopyranoside, were isolated from leaves of Ruelliapatula JACQ., together with 12 known compounds, β-sitosterol glucoside, vanilloside, bioside (decaffeoylverbascoside), acteoside (verbascoside), syringin, benzyl alcohol O-β-Dxylopyranosyl-(1íí,2í)-β-D-glucopyranoside, cistanoside E, roseoside, phenethyl alcohol O-β-Dxylopyranosyl-(1íí,2í)-β-D-glucopyranoside, (+)- lyoniresinol 3-α-O-β-D-glucopyranoside, isoacteoside and 3,4,5-trimethoxyphenol O-α-L-rhamnopyranosyl-(1íí,6í)-β-D-glucopyranoside. Their structures were elucidated by means of spectroscopic analyses.

MATERIALS AND METHODS

CollectionandAuthenticationofPlants

The flower of *R.brittoniana*were collected in and around Depok, West Java, Indonesia the month of November and December. The plants were authenticated by Prof. P Jayaraman, Ph.D Director ,Professor Presidency college Chennai. Voucherspecimens have been kept in our laboratory for future reference.

Preparationofextract

The flower of *R.brittoniana*were dried in shade and pulverized to a coarse powder.The coarse powders (2 Kg) were subjected to continuous hot extraction in soxhlet apparatususingthealcohol (95% v/v).

Materials

Shadedriedflower powderof *R.brittoniana*

Alcohol(95% v/v)

EXTRACTION PROCESS:

Alcoholextract

The coarse powders of *R.brittoniana*were extracted with 2-3 litres of ethanol (95% v/vC)by continuous hot percolation using Soxhlet apparatus.The extracts were evaporated todryness under vacuum.The extract was evaporated to dryness under vacuum.The dried extracts were stored in vacuum desiccators.The yield of the extract of the plant studied is shown in **Table1.**

Table1

Data Showing the Extractive Values of flower of *R.brittoniana*

Plantname	**Partused**	**Methodof extraction**	**Yield in percentage(%)**
R.brittoniana	Flower	Continuous hot percolation	4

PRELIMINARYPHYTOCHEMICALSTUDIES[46-47]

The alcohol extract of the flower of *R.brittoniana* were subjected to phytochemical tests for the identification of their active constituents as described.

1. TESTS FOR CARBOHYDRATES AND GLYCOSIDES

A small quantity of the extract were dissolved separatel in 4ml of distilled water and filtered .The filtrate was subjected toMolisch'stest todetect the presence of carbohydrates.

Molisch's Test

Filtrate was treated with 2-3 drops of 1% alcoholic α-naphthol solution and 2ml of con. H_2SO_4 was added along the sides of the test tube. Appearance of violet coloured ring atthe junction of two liquids shows the presence of carbohydrates.

Another portion of the extract was hydrolysed with HCl for few hours on a water bath and the hydrolysate was subjected to Legal's and Borntrager's test to detect the presence of different glycosides.

Legal's Test

To the hydrolysate, 1ml of pyridine and few drops of sodiumnitroprusside solution were added and then it was made alkaline with sodiumhydroxide solution. Appearance of pink to red colour shows the presence of glycosides.

Borntrager's Test

Hydrolysate was treated with chloroform and then the chloroform layer was separated. To this equal quantity of dilute ammonia solution was added. Ammonia cal layer acquire spink colour showing the presenceof glycosides.

2. TESTS FOR ALKALOIDS

A small portion of the solvent free Pet. ether and alcohol extracts were stirred separately with few drops of dil.HCl and filtered. Thefiltrate was treated with various agents as shown for the presence of alkaloids.

Mayer'sreagent - Creamy precipitate

Dragandroff'sreagent - Orange brown precipitate

Hager'sreagent - Yellow precipitate

Wagner'sreagent - Reddishbrownprecipitate

3. TESTS FOR PHYTOSTEROL

The extract was refluxed with solution of alcoholic potassiumhydroxide till complete saponification takes place.The mixture was diluted and extracted with ether.The ether layer was evaporated and there is due wastested for the presence of phytosterol.

Libermann Burchard Test

There is due was dissolve few drops of aceticacid, 3drop so faceti can hydride was added followed by few drops of con. H_2SO_4. Appearance of bluish green colour shows the presence of phytosterol.

4.TESTS FOR FIXED OILS

Spot test

Small quantities of various extracts were separately pressed between two filterpapers. Appearance of oil stain on the paper indicates the presence of fixed oil.

Few drops of 0.5N alcoholic potassium hydroxide were added to a small quantity ofvarious extracts along with a drop of phenolphthalein. The mixture was heated on a water bath

for 1-2 hours. Formation of soap or partial neutralization of alkali indicates the presence offixed oils and fats.

4. TESTS FOR GUMS AND MUCILAGES

Small quantities of the extracts were added separately to 25ml of absolute alcohol with constant stirring and filtered.The precipitate was dried in air and examined for its swelling properties for the presenceof gums and mucilages.

6. TESTS FOR SAPONINS

The extract was diluted with 20ml of distilled water and it was agitated in agraduated cylinder for 15 minutes. The formation of 1cm layer of foam shows the presence ofsaponins.

7.TESTS FOR PROTEINS AND FREE AMINOACIDS

Small quantities of the extracts were dissolved in few ml of water and treated withfollowing reagents.

1. Millon's reagen - Appearance of red colour shows the presence of protein and free amino acids.
2. Ninhydrin reagent - Appearance of purple color shows the presence of proteins and free amino acids.
3. Biuret test - Equal volumes of 5% NaOH solution and 1% copper sulphate solution were added. Appearance of pink or purple colour shows the presence of proteins andfree amino acids.

8.TESTS FOR PHENOLIC COMPOUNDS AND TANNINS

Small quantities of the extracts were taken separately in water and tested for the presenceof phenolic compounds and tannins using following reagents.

1. Dil.$FeCl_3$ solution (5%) - violet colour
2. 1% solution of gelatin containing 10% NaCl - white precipitate
3. 10% lead acetate solution - white precipitate.

9.TESTS FOR FLAVONOIDS

1. With aqueous Sodium hydroxide solution

Blue to violet colour (anthocyanins), yellow colour (flavones), yellow to orange(flavonones)

2. With Con.H2SO4

Yellow orange colour (anthocyanins), yellow to orange colour (flavones), orange tocrimson (flavonones)

3. Shinoda'stest

Small quantity of the extract was dissolved in alcohol and to that a piece of magnesium followed by Con. HCl drop wise were added and heated. Appearanceof magenta colour shows the presence of flavonoids.

The results of preliminary phytochemical studies of *R.brittoniana* are presented in

Table 2.

CYTOTOXICITYSTUDIES

Tissue culture has been used to screen may anticancer drugs since there is clear correlation between the *in vitro* and *in vivo* activities of potential chemotherapeutic agents. There is scientific justification for cytotoxicity testing in tissue culture, since animal models are in many ways inadequate for predicting the effects of chemicals on humans since there aremany metabolic differences between species. Cytotoxicity studies involve the analysis of morphological damage or inhibition of zone of outgrowth induced by the chemicals tested..

ASSAY FOR PROLIFERATION STUDIES

MTT(3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) method

MTT measures the metabolic activity of viable cells. The assay is non-radioactive and can beperformed entirely in a micro titer plate (MTP). It is suitable for measuring cell proliferation,cell viability and cytotoxicity. This method is based on the principle that viable cells convertMTT into a formazan salt, which is insoluble. It is solublised and quantified. Increase in itsconcentration indicates increased number of viable cells. The absorbance directly correlates with the cell number. This methodisapplicable for adherent cells culture din MTP.

Materials for MTT assay

- RPMI-1640media(Himedia,Mumbai,India)
- Fetalbovineserum (Gibco's,USA)
- Penicillin-G (Himedia,Mumbai,India)

- Streptomycin (Himedia,Mumbai,India)
- Amphotericin B (Courtesy, Dr.V.Venkateshwarlu, UiversityCollege of Pharmaceutical Sciences, Kakatiya University,Warangal,A.P.,India)
- Phosphate buffered saline (PBS) (Himedia,Mumbai,India)
- Trypsin (Trypsin-EDTA[1x]inHBSS,Gibco'sUK)
- Ethylenediaminetetra-aceticacid(EDTA)(Himedia,Mumbai,India)
- Trypanblue (Himedia,Mumbai,India)
- Dimethylsulphoxide (DMSO)(MerckIndiaLtd,Mumbai,India)
- SDS lysis buffer(Himedia,Mumbai,India)
- MTT(3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazoliumbromide) (Himedia,Mumbai, India)

Additionalequipmentsrequired

- CO_2 incubator (WTCBinder,Germany)
- Laminar air flow cabin (Klenzaids,Chennai,India)
- Refrigerated centrifuge (Biofuge Fresco,byHeraeus,Germany)
- ELISA-reader (for MTP) (Anthos2010,Germany)
- Deep freezer (Polar Angelant on iIndustries,Italy)
- Ultrasonicbath (Transonic[460/H],byElma®,Germany)
- Vaccumpump (Zenith[model:PDF-2-2.5],Mumbai,India)
- Pipettes (Eppendorf,Hamburg,Germany)
- Cultureplates (Felcon,Germany)
- Centrifugetubes (Felcon,Germany)

- Eppendorf tubes (Tarsons,Mumbai,India)
- Aerosol resistant tips (Tarsons,Mumbai,India)
- Flat-bottomed 96-well MTP,tissue culture grade(Tarsons,Mumbai,India)

Procedure:

Preparation of antibiotic solutions

Penicillin-G

- 0.61g (onevial) of penicillin-G was weighed and dissolved in 1ml of sterile phosphate buffered saline(PBS).
- Contents were stirred for 5 minutes.
- The contents were sterilized with syringe by passing through 0.22micron f ilter aliquot of 5 ml fractions in 15 ml storage vials.
- Stored at–20^0 C until use.

Streptomycin sulfate

- 10g (onevial) of streptomycin sulfate was weighed and dissolved in 10ml of sterile PBS.

Amphotericin-B

- 250mg of Amphotericin-B was weighed and dissolved in10ml of sterile PBS.

Preparation of Dulbecco's PBS

- 9.6 g (one vial) of D-PBS was suspended in 800 ml triple distilled water and mixeduntil dissolved.
- It was then autoclaved at 121^0 C (15 lbl) for 15 minutes.
- After cooling to room temperature aseptically, sterile 100ml $CaCl_2$ (1mg/ml) solutionand 100 ml $MgCl_2$ (1mg/ml) solution were added and mixed.

Preparation of Lysis buffer

- 15 g of sodium chloride was weighed and dissolved in 1:1 mixture of dimethylformamide and distilled

water. The final pH was adjusted to 3 with ortho- phosphoric acid.

Preparation of RPMI-1640 Medium

- 10.39 g (one vial) of RPMI-1640 was weighed in a sterile conical flask.
- The contents were dissolved in 900 ml of Milli-Q (triple distilled) water by stirring and the antibiotic solutions were added (100 U/ml Penicillin-G Sodium, 50µg/ml of streptomycin and 2 µg/ml of Amphotericin-B)
- Once all constituents of the medium were completely added, the pH was adjusted to 7.2to 7.5 with 0.1 N HCl.
- The volume was made up to one liter with triple distilled water.
- Contents were transferred to the Millipore filtration kettle (Duran, Germany) supportedby 0.22 microns membrane filter, kept in laminar flow hood that was connected to the outlet by negative pressure pump and filtered.
- Pressure was adjusted such that the flow rate of medium from the filtration unit is 10- 12 min/L i.e., 100 ml/min
- 5 ml of the sample is taken in T_{25} tissue culture flask; kept for 24 hours in CO_2 incubator for sterility check.
- 100 ml of sterile Fetal Bovine Serum was added to 900 ml medium.
- Reconstituted medium was checked for sterility by transferring 5ml of medium into a T_{25} tissue culture flask and incubated for 24 hours in CO_2 incubator.

Trypsinization

- The media was aspirated from each flask, being sure to change pipettes between celllinesto prevent crosscontamination.
- Monolayer was rinsed with 5-10 ml PBS to remove traces of serum and the rinsingsolution wasaspirated.

- 1 ml of 0.25 % trypsin-EDTA (Himedia) was added to each flask and spread evenlyover cell monolayer and depending on cell type, the flask was either placed in hood orinincubator for 2-5 minutes.
- Theflaskwasgently‘tapped’fordislodgingthe cells.
- Then the cells were resuspended in 8 ml of the medium containing serum to stop theaction of the trypsin. Gentle pippetting was carried out up and down for breaking upthe clumps.
- Cellsuspensionwastransferredtoa properlylabelled15mlcentrifuge tube.
- The tubeswerecentrifugedat 1000 rpm for 5 minutes.
- Thepelletwasresuspendedin5-10mlofmediumdependinguponthesizeofthepelletorcell number.

MTT assay[48-49]

Principle

MTT[(3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazoliumbromide]measuresthemetabolic activity of the viable cells. The assay is non-radioactive and can be performedentirely in a microtiterplate (MTP). It is suitable for measuring cell proliferation, cell viabilityor cytotoxicity. The reaction between MTT and ‘mitochondrial dehydrogenase’ produceswater-insolubleformazansalt.Thismethodinvolvesculturingthecellsina96-wellmicrotiterplate, and then incubating them with MTT solution for approximately 2 hours.During incubation period, viable cells convert MTT to a water-insoluble formazan dye. Theformazan dye in the MTP is solubilized and quantified with an ELISA plate reader. Theabsorbance directly correlates with the cell number. This is applicable for adherent cellsculturedinMTP.

Materials

RPMI-1640, (Himedia, Mumbai India)TRYPSIN-0.25%(GibcosUSA)

FBS (Fetalbovineserum) (GibcosUSA)

MTT 4mg/ml (Himedia)

DMSO (Emerck India)

Lysis buffer (15% SL Sin 1:1DMF and water)

Composition of RPMI; 9.54g/lit, 10%FBS ,2000mg so diumbi carbonate, 250µl each of penicillin (60mg/ml), streptomycin (100mg/ml), Amphotericin (200mg/ml)

Celllinesused

- ❖ HeLa-Human Cervical Adenocarcinoma cells (National Center for Cell Sciences,Pune, India)

METHOD

0.1ml of the cell suspension (containing 5×10^5cells/100µl) and 0.1ml of the testsolution (10µg - 300 µg in DMSO such that the final concentration of DMSO in media is lessthan 1%) were added to the 96 well plates and kept in corbondioxide incubator with 5% CO_2, at 37^0C for 72hours.Blank contains only cell suspension and control wells contain 1%DMSO and cell suspension

After 72 hours, 20µl of MTT was added and kept in a corbondioxide incubator for 2 hours followed by 80µl of lysis buffer (15%SLSin 1:1DMFand water). The plate was covered with aluminum foil to protect it from light. Then the 96 well plates are kept in rotary shaker for 8hours

After 8 hours, the 96 well plates were processed on ELISA reader for absorption at 562nm. The readings were averaged and viability of the test samples were compared with DMSO control

The percentage growth inhibition was calculate dusing the following formula

$$\%\text{Growth Inhibition} = 100 - \frac{\text{Mean OD of Individual Test Group}}{\text{Mean OD of Control Group}} \times 100$$

Trypan blue dye exclusion assay[50]

Principle

Trypan blue is a vital stain used to selectively colour dead tissues or cells blue. It is adiazo dye. Trypan blue is recommended in dye Exclusion procedures for viable cell counting based on the principle that live (viable) cells actively pump out the dye by efflux mechanism where as dead (non-viable) cells do not. So in this assay whit etrans parent cells are viable cells and blue cells taking up the dye are dead cells.

Celllineused

HeLa - Human cervical Adenocarcinoma Cancer cells

Procedure:

1. Cel l suspension were prepared in a medium.
2. Transferred 700µl of a cell suspension to24wel l plate and incubate 24hrs in 5%CO_2.

After incubation add 300µl of drug with varying concentration of *Ruelliabritoniana* extract (62.5-1000µg/ml) and incubate 48 h.

3. Take 100 µl of cell suspension and add 100 µl of 0.4% Trypan Blue Solution to a Eppendorf tube and mix thoroughly. Allow to stand for 5 to 15 minutes.
4. With the cover-slip in place, use a Pasteur pipette to transfer a small amount of trypan blue-cell suspension mixture to both chambers of a hemocytometer. Carefully touch the edge of the cover-slip with the pipette tip and allow each chamber to fill by capillary action.
5. Starting with chamber 1 of the hemocytometer, count all the cells in the 1 mm center square and four 1 mm corner squares. Non-viable cells will stain blue. Kept a separate count of viable and non-viable cells.

Calculation

$$\% \text{ Inhibition} = \frac{\text{Numberofnonviablecells(Stained)}}{\text{TotalCells(StainedandUnstained)}} \times 100$$

RESULTS AND DISCUSSION

Cancer is basically a disease of cells characterised by a shift in the control mechanisms that govern cell proliferation and differentiation. Cells that have undergone neoplastic transformation usually express cell surface antigens that appear to be of normal fetal type and have other signs of apparent "immaturity" and may exhibit qualitative or quantitative chromosomal abnormalities, including various translocations and the appearance of amplified gene sequences[51]. Such cells proliferate excessively and form local tumors that can compressor invade adjacent normal structures.

Next to cardiac diseases, cancer is the major cause of death in the world. With present methods of treatment, one third of patients are cured with local measures (surgery or radiation therapy), which are quite effective when the tumor has not metastasized by the time of treatment[52]. Earlier diagnosis may lead to increased cure of patients with such local treatment; however in the remaining cases early micrometastasis is a characteristic feature of the neoplasm, indicating that a systemic approach such as can be attained with chemotherapy will be required (often along with surgery or radiation) for effective cancer management. At present, about 50% of patients with cancer can be cured with chemotherapy, contributing to cure in about 17% of patients.

Search for anticancer drugs

Cancer chemotherapy as currently employed can be curative in certain disseminated neoplasms that have undergone either gross or microscopic spread by the time of diagnosis. Drug research may provide the ultimate cure for cancer, although many of the currently available agents fall short of this goal. A major effort to develop anticancer drugs through both empirical screening and rational design of new compounds has now been under way for three decades.

Ideal anticancer drugs would eradicate cancer cells without harming normal tissues. Unfortunately, no currently available agents meet this criterion and clinical use of these drugs involves a weighing of benefits against toxicity in a search for a favourable therapeutic index[53].

Role of chemotherapy in cancer

Prevention is undeniably the sensible maneuver towards the ultimate goal of cancer control[54]. Several methods exist for the treatment of cancer in modern medicine. These include chemotherapy, radiotherapy and surgery. Chemotherapy is now considered as the most effective method of cancer treatment. Intervention with chemopreventive agents at the early stage in carcinogenesis is theoretically more rational than attempting to eradicate fully developed tumours with chemotherapeutic drugs. However, most cancer chemotheraputants severely affect the host normal cells[55]. Hence, the use of natural products now has been contemplated of exceptional value in the control of cancer and its eradication programme[56].

Choice of plants for the present study

Efforts are being made to develop anticancer agents from natural sources. In the past, plants have provided effective anticancer compounds like Vincristine and Taxol. Based on the ethnopharmacological information available *Ruellia Britoniana* (Family: Acanthacea) have been selected for the present study. An attempt has been made to evaluatethe *in vitro* cytotoxicity studies of these plant on various antiproliferation assay models..

Phytochemicalstudies

Phytochemical analysis is intended to screen, identify, extract and isolate the phytoconstituents to evaluate the therapeutic potential of the plant and to develop phytochemical standards forthe medicinal plant materials for quality control purpose. The preliminary phytochemicalstudies on EIA indicate the presence of alkaloid, glycoside, saponin, tannin, phytosterol and flavonoid. These results are shown in the following **table**

Table 2

Data Showing Preliminary Phytochemical Screening of the ethanol extract of *RuelliaBrittoniana*

Plant	Alkaloids	Carbohydrates	Glycosides	Fixed oils&Fats	Gums& Mucilages	Proteins &aminoacids	Saponins	Tannins	Phyto sterol	Flavonoids
RuelliaBritoniana	+	+	+	+	-	-	+	+	+	+

\+ =Present

-- =Absent

Cytotoxic effect of *Ruellia Brittoniana* by MTT assay

Cytotoxicity studies have been used to screen many anti-cancer drugs since their demonstration in 1950 of a clear correlations between the *in vitro* and *in vivo* activities of potential chemotherapeutic agents. Explants of human tumour tissues grown *in vitro* can be tested for chemo sensitivity in order to tailor the patient's chemotherapy to suit the individual patient and tumour. Cytotoxicity studies involve the analysis of morphological damage or inhibition of zone of outgrowthinduced by the chemical being tested.

Cytotoxicity is one of the chemotherapeutic targets of antitumour activity. Cytotoxicity screening models provide important preliminary data to help in the selection of compounds or plant extracts with potential antineoplastic properties for further studies[57]

Human tumor cells were obvious subjects for research; human tumor cells could give rise to continuous cell lines. The first human cell line to be grown continuously in the laboratory was the HeLa cell line, which was obtained from a malignant adenocarcinoma of the uterine cervix. This cell line opened up the possibility of

characterizing the cells of malignant tumors *in vitro* and is still one of the most popular human cell lines for study.

The four most common cancers, accounting for more than half of all cancer cases, are those of the prostate, breast, lung, and colon / rectum.

Presently 60 different human tumour cell lines are available for screening malignancy such as leukemia, melanoma and cancer of lungs, colon, brain, ovary, breast, prostate and kidney. The aim of the test is to characterize compounds showing selective growth inhibition or killing of particular tumour cell lines[57].

Scientific strategies for the *in vitro* evaluation of natural products with biological activity havechanged in the past few years. One recent development is the highly automated bioassay screening based on colorimetric methods that quantify the proliferation of cell cultures. Many biological assays require the measurement of surviving and/or proliferating mammalian cells. This can be achieved by MTT assay, a non –radioactive, fast and economical assay widely used to quantify cell viability and proliferation[57].

MTT is a yellow water soluble tetrazolium salt. Metabolically active cells are able to convert the dye to water insoluble dark blue farmazan by reductive cleavage of the tetrazolium ring. MTT is cleaved by all living, metabolically active cells but not by dead cells or erythrocytes. The amount of farmazan generated is directly proportional to the cell number over a wide range, using a homogenous cell population.

The results of the cytotoxic activity test of ethanol extract of *R. brittoniana* flowers on HeLa cervical cancer cells are presented in **Figure 1**. The graph in **Figure 1** shows that there is a positive relationship between the log concentration of ethanol extract and percentage of inhibition on HeLa cervical cancer cell, it means that the higher log concentration, the higher percentage of inhibition on HeLa cells (**Figure 2**). Therefore, the largest percentage of inhibition, ie 67.8%, occurs in administration of a maximum ethanol extract concentration of 200 ppm. The linear regression equation from the graph is y = 42.148x-37.099. The antilog value of the x coefficient is proportional to the IC_{50} value, which is the concentration needed to inhibit 50% growth of HeLa cervical cancer cells.

Based on the calculation of the linear regression equation, the IC_{50} value of ethanol extract was found to be **116.55**

ppm on HeLa cell lines.

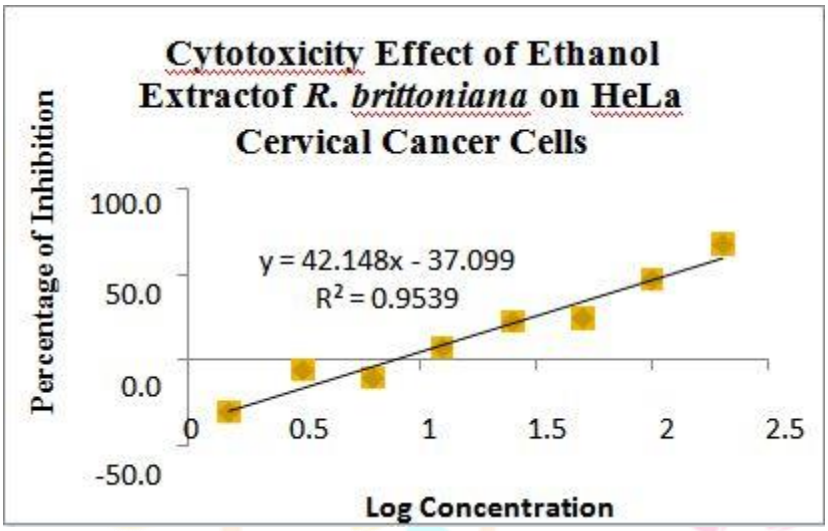


Figure 1

Relationship between log concentration and percentage of inhibition of ethanol extract of R. brittoniana on HeLa cervical cancer cells.

Figure 2

Anticancer activity evaluation of ethyl acetate extract of *R. brittoniana* flowers on HeLa cervical cancer cells are

presented in Figure 3. The graph in Figure 1 shows that the higher log concentration of ethyl acetat extract of *R. brittoniana* flower, the higher percentage of inhibition on HeLa cells. Percentage of inhibition increased from -7.0% until 67.9% as concentration increases. The linear regression equation from the graph is y = 39.346x - 17.72. Based on the calculation through linear regression equation, the IC_{50} value of ethanol extract is 52.62 ppm. Anticancer activity of n-hexane extract of *R. brittoniana* flowers on HeLa cervical cancer cells are presented in **Figure 4**. The graph in **Figure 3** shows that percentage of inhibiton on HeLa cervical cancer cell would be higher as the log concentration increases. The highest percentage of inhibition is 68,9%, which is occurs in administration of a maximum concentration of n-hexane extract at 200 ppm. The linear regression equation from the graph is y = 35.727x- 24.678. Based on the calculation through the linear regression equation, the IC_{50} value of ethanol extract is 123.09 *ppm*.

Among three extracts of *R. brittoniana* flowers, ethyl acetate extract has the smallest IC_{50} value. This suggesting that ethyl acetate extract has the strongest anticancer activity against HeLa cells compared to etanol and hexane extracts. Meanwhile, n-hexane extract has the largest IC_{50} value, which indicating that n-hexane extract has lowest ability to inhibit a growth of HeLa cells. In this study, cisplatin as a positive control, has an IC_{50} value of 1.78 ppm. Compared to extracts of *R. brittoniana* flowers, cisplatin has a smaller IC_{50} value, this shows that the three extracts of

R. brittoniana have a weaker ability to inhibit the growth of HeLa cells compared to cisplatin.

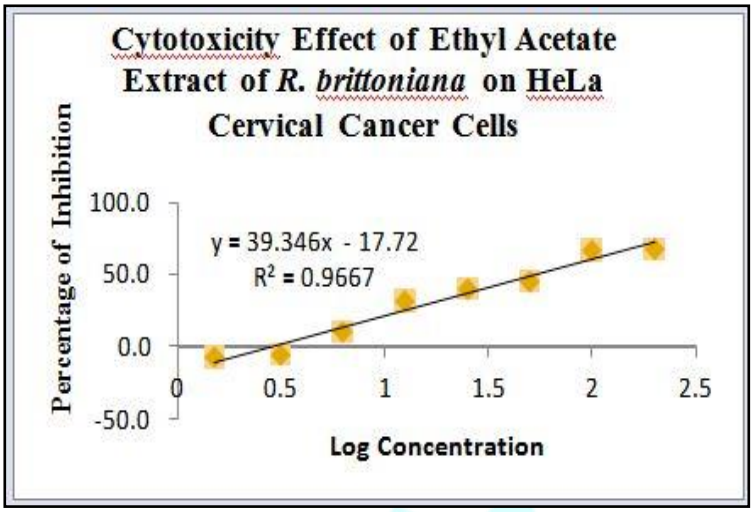


Figure 3

Relationship between log concentration and percentage of inhibition of ethyl acetate extract of *R. brittoniana* on HeLa cervical cancer cells.

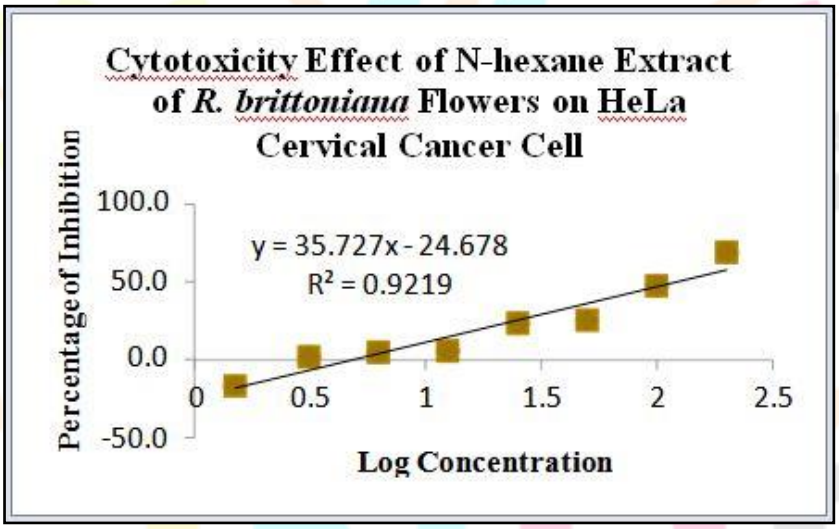


Figure 4

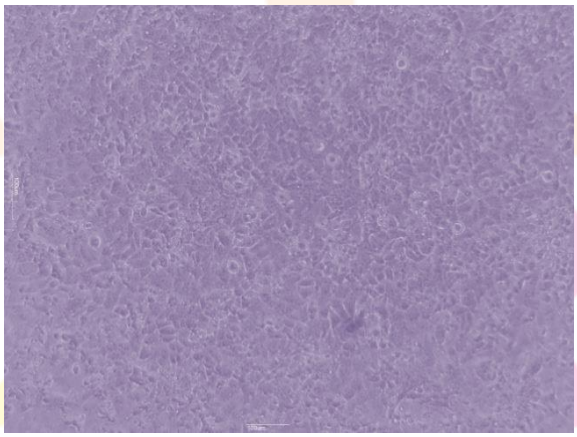
Relationship between log concentration and percentage of inhibition of n-hexane extract of R. brittoniana on HeLa Cervical cancer cells.

Cytotoxicity effect of *RuelliaBritoniana* on HeLacells by MTTassay.

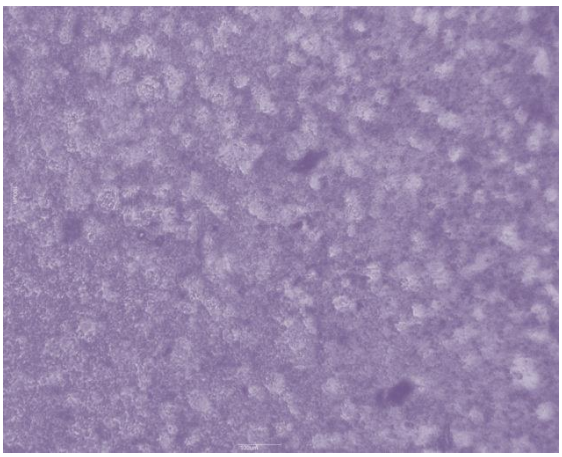
Sl.No.	Name of TestSubstances	TestConc.(μg/mL)	Log con	% Cytotoxicity	CTC50 (ppm)
1	*RuelliaBritoniana*	1000	4	91.50±0.5	**116.55*ppm***
		500	3.699	88.98±0.5	
		250	3.398	79.09±1.2	
		125	3.097	67.20±0.3	
		62.5	2.796	35.40±1.1	
		31.25	1.995	23.89±1.4	
		15.625	1.054	15.38±1.4	
		7.8	0.42	8.27±0.2	

Average of 3 determinations , 3replicates

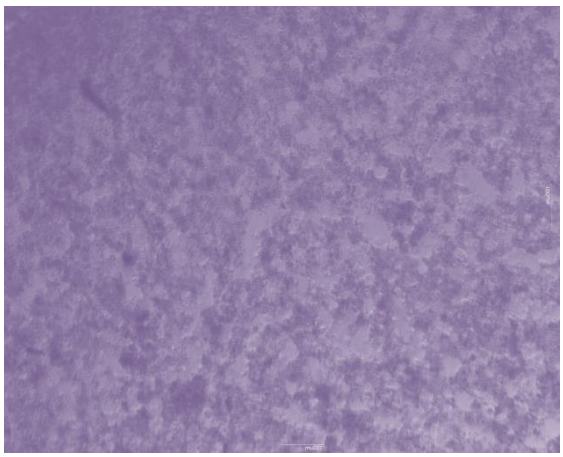
IC_{50},drug concentration inhibiting 50% cellular growth following the drug exposure



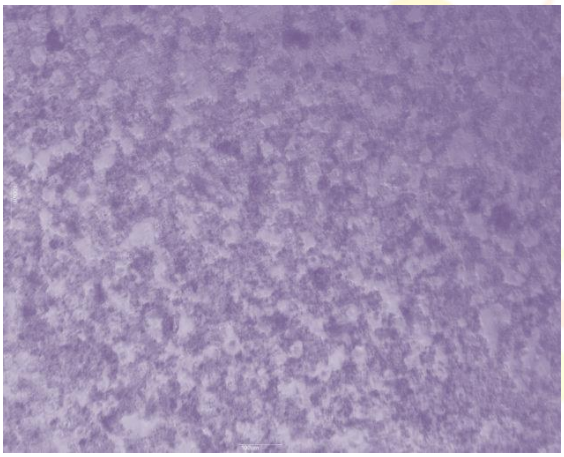
Control



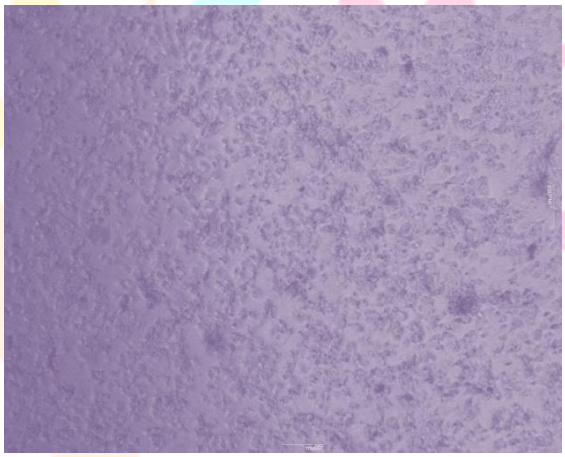
1000µg/mL



500µg/mL

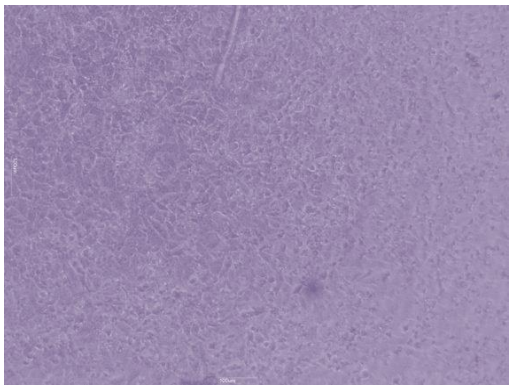


250µg/mL

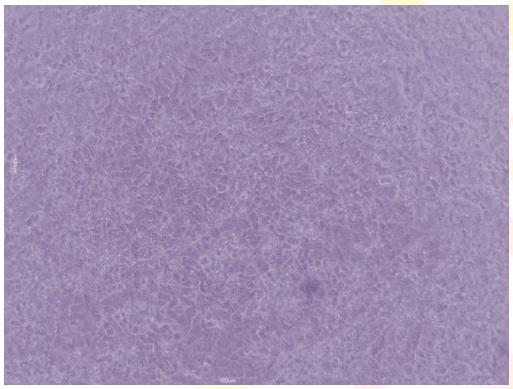


125µg/mL

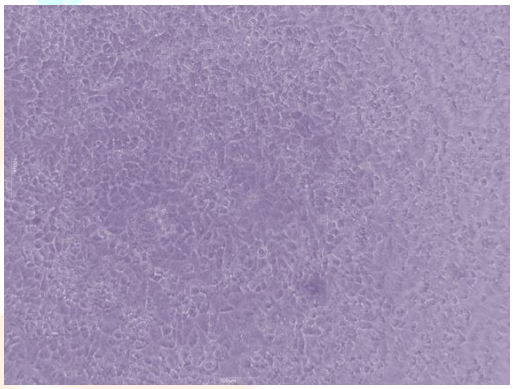
62.5µg/mL



31.25µg/mL



15.625µg/mL



7.8µg/mL

Morphological Effect of *RuelliaBritoniana* on HeLacells

Morphological studies results of *Ruellia Britoniana* on HeLa cell line shows the followingcharacterisations.

The picture of morphological changes were presented in Figure above.

Cell control:

Normal cells of HeLa cell line with full confluency; no growth inhibition; no detachment or cell lysis.

1) <u>Test substance:</u>

a) **1000 µg/mL** –

Nearly complete destruction of the cell layer. 90% of the cells were dead. Reactivity is concluded as severe.

b) **500 µg/mL –**

Nearly complete destruction of the cell layer. 87% of the cells were dead. Reactivity is concluded assevere.

c) **250μg/mL-**

Cell layer was notc ompletely destroyed, but more than 70% growth inhibition was observed. 78% of the cells were dead. Reactivity is concluded as moderate.

d) **125μg/mL-**

Cell layer was not completely destroyed, but more than 50% growth inhibition was observed. 66%of the cells were dead. Reactivity is concluded as moderate.

e) **62.5 μg/mL –**

No extensive cell lysis; not more than 50% growth inhibition was observed. 35% of the cells were dead. Reactivity is concluded a smild.

f) **31.25μg/mL-**

Lesslysed cells were present; only slight growth inhibition was observed. 23% of the cells were dead. Reactivity is concluded as slight.

g) **15.625 μg/mL –**

Very less lysed cells were present; only slight growth inhibition was observed. 15% of the cells were dead.Reactivity is concluded as slight.

h) **7.8 μg/mL –**

No lysed cells were present; no growth inhibition was observed. 7% of the cells were dead. Reactivity is concluded as none.

The morphological results of HeLa cell observed in MTT assay shows ERB having significant cytotoxicity effects.

TRYPHAN BLUE DYE EXCLUSION TECHNIQUE

Cytotoxicity studies by trypan blue exclusion method is a very simple method which can be carried out within a short time of 48 hrs. It is a precise method, which takes in to account the viable and also the dead cells in addition to estimation of IC_{50}concentration.

The$IC_{50}$of ***RuelliaBritoniana*** was found to be170 µg/ml against **HeLa** cell lines.

Cytotoxicity studies of *RuelliaBritoniana*on HeLa cells by Tryphan Blue Dye Exclusion Technique

Concentrations (µg/ml)	**MPC % cell death**
1000	88.65 ±2.0
500	68 ±1.10
250	56 ±1.4
125	34 ± 2.5
62.5	17.4 ±3.0
IC_{50}(µg/ml)	**170**

Average of 3 determinations,3replicates

IC_{50}, drug concentration inhibiting 50% cellular growth following the drug exposure

From the results, it can be concluded that the ERB is selectively toxic against HeLa celllines.The IC_{50} value for the ERB was found against HeLa cells (116.55ppm & 200 mg/ml) in the MTT assay and Tryphan blue dyeexclusion technique.

.Flavonoids are known to possess antimutagen and antimalignancy effect[58]**.** In addition toantineoplastic activity, flavonoids exert growth inhibitory effects on several malignant tumour celllines*in vitro*[59-60].

Moreover, flavonoids have a chemopreventive role in cancer through signal transduction in cell proliferation[61]and angiogenesis[62]. The list of flavonoids with provenantineo plastic effectis longone,which includes the following

1. Isoflavonoids-tectorigenin, tectoridenandgenisteinfrom*Pueraria thunbergiana.*[63]
2. Lavandulylate flavanones from *Sophorol flavescens*[64]
3. Flavonesacacet in and luteolin from *Chromolaenaodorata*[65]

Flavonoids may inhibit carcinogenesis by actingas “blockingagents” by one or more of the following possible mechanism.

Inhibiting the metabolic activation of the carcinogen toits reactive in termediates.

1. Inducing the enzymes involved in thedetoxification of the carcinogen and

2. Binding to reactive form so carcinogens, there by preventing their interaction with critical cellular targets such as DNA, RNA and proteins.

In addition, plant flavonoids could also inhibit tumour promotional events as mentioned above. It is likely that flavonoids may emerge as a distinct group of antitumour agents. Based on previous reported scientific studies, our results show the presence of flavonoids in phytochemical studies. Hence present the present study suggest that cytotoxic effect of EIA against HeLa cell line may due to presence of flavonoids. Further study needed in near future to isolate specific phytochemicals and same subjected to experimental studies against various cancer cell lines.

SUMMARYANDCONCLUSIONS

- *RuelliaBritoniana (Family:Acanthaceae)* has been selected for invitro cytotoxicity studies against Hela cells using various antiproliferation assay models.
- The presence of alkaloid, glycoside, saponin, tannin, phytosterol, and flavonoid has beendiscoveredin preliminary phytochemicalstudieson ERB.
- The ethanol extract of Britoniana was studied for its cytotoxicity properties against human cervical adenocarcinoma cancer cell lines [HeLa] by the MTT assay and the trypan blue exclusion method, respectively.
- The IC_{50} of the ethanol extract of *RuelliaBritoniana* was found to be116.55 ppm and170mg/mlagainst HeLacell lines.
- The result was found to be that RuelliaBritoniana possessed cytotoxicity properties against human cervical adenocarcinomacells.

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