The Preclinical Determination of Antineoplastic Activity of *Colocasia gigantea* (Blume) Hook. *f.* *in Vitro* and *in Vivo*

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CERTIFICATE

This is to certify that **Smt. Nambam Bonika Devi** carried out her research work under my supervision since 2014. The thesis entitled **"The Preclinical Determination of the Antineoplastic Activity of *Colocasia gigantea* (Blume) Hook. *f. in Vitro* and *in Vivo*"** is an original piece of work and has not been submitted for any other degree of any other university.

Place: Aizawl
Date: 31 May 2018

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DECLARATION

I, Nambam Bonika Devi, hereby declare that the subject matter of this thesis entitled **"The Preclinical Determination of the Antineoplastic Activity of *Colocasia gigantea* (Blume) Hook. *f. in Vitro* and *in Vivo.*"** is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the Mizoram University, Aizawl for the award of the degree of Doctor of Philosophy in Zoology.

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Affectionately Dedicated to…

ESTEEMED GUIDE
&
MY LOVING AND CARING
HUSBAND & DAUGHTER

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ABBREVIATIONS

CGC	Chloroform extract of *Colocasia gigantea*
CGE	Ethanol extract of *Colocasia gigantea*
CGA	Aqueous extract of *Colocasia gigantea*
ABTS	2,2-azinobis (3-ethylbenzothiazoline-6-sulphonic acid)
CDNB	1-Chloro 2,4-Dinitrobenzene
DPPH	2, 2-diphenyl-1-picryl hydrazine
DTNB	5-5'-dithiobis[2-nitrobenzoic acid]
EDTA	Ethylenediamine tetra-acetic acid
MTT	(3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide)
NADH	b-nicotinamide adenine dinucleotide
NBT	Nitroblue tetrazolium
PMS	Phenazine methosulphate
ROS	Reactive oxygen species
TBA	Thiobarbituric acid
TCA	Tricarboxylic acid
TLC	Thin layer chromatography
DLA	Dalton's lymphoma ascites
DOX	Doxorubicin
MNM	Mononucleated micronucleus
BNM	Binucleated micronucleus
AST	Average Survival Time
MST	Median Survival Time
IALS	Increase in Average Life Span

IMLS	Increase in Median Life Span
GSH	Glutathione
GST	Glutathione-S-Transferase
CAT	Catalase
SOD	Superoxide Dismutase
LOO	Lipid Peroxidation
ALT	Alanine Aminotransferase
AST	Aspartate Transaminase
SPS	Saline Physiological Solution
µg	Microgram
Rf	Retention Factor

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1

Cancer is a Latin word, which literally means crab and it is known as *karkinos* in the Greek language, which also means crab. The term cancer was first used by Hippocarates, the father of modern medicine, who observed that the blood vessels around malignant tumors looked like the crab claws and coined the term cancer. Cancer is one of the most common and severe diseases in clinical medicine, which a group of diseases used to describe more virulent forms of neoplasia. Cancer is a disease process characterized by uncontrolled cellular proliferation leading to a mass of cells that is known as cancer or tumor. The cancer is one of the leading cause of death in the developed world, which is second to cardiovascular diseases (Siegel *et al*., 2016). Cancer is a group of diseases that comprises mutation or dynamic changes in the genome of the cell producing proteins that disturb the normal cellular balance leading to the uncontrolled proliferation of cells (Bishop and Weinberg, 1996; Hejmadi, 2010; American Cancer Society, 2015). In normal condition, the cells enter the active proliferative phase only after receiving the mitogenic growth signals, and cannot multiply in the absence of these signals. However, these stimulatory signals are deregulated in cancer cells and therefore cancer cells are able to proliferate even in the absence of these signals (Hanahan and Weinberg, 2000). Cancer cells have the ability to produce their own growth factors mimicking the normal growth factors which make them independent of the normal growth factors (Fedi *et al*., 1997).

All forms of cancer involve out-of-control growth and formation of a mass of abnormal cells into a tumor. The normal cells in humans divides only to replace worn-out or damaged or dying cells and/or to repair injuries, whereas the cancer cells continue

to grow and divide defying all the regulatory mechanisms of cell growth and division. They also have the propensity to spread into the other parts of the body also known as invasion or metastasis. The cancer or neoplastic cells accumulate and form tumors (lumps) that may compress, invade, and destroy normal tissue/s. The neoplastic cells may break away from such a tumor, invade the blood or lymph vessels and can travel through the blood stream or the lymph system to other areas of the body, where they may settle, colonize and form tumors. In their new location, the cancer cells continue to grow forming metastases which are still named after the part of the body from they originated (Woodhouse, 1997; Liotta, 1991; Leberand Efferth, 2009).

There are different types of cancer based on the part of the body where it begins and by its appearance under a microscope and thus varies according to their growth rates, patterns of spread and response to different types of treatment. Therefore, people with cancer needs to aimed at their specific form of the disease. The lifestyle and diet also plays a very important role in the causation of cancer (WHO, 2003).

Though cancer can arise due to mutation, a single mutation is not enough to cause cancer. Amplification of the accumulated DNA mutation is required for the development of cancer (Hejmadi, 2010). The evolutionary concept "Survival of the fittest" applies in case of carcinogenesis as well as due to the multiple checks and balances that exist in stem cells to limit excess cell division. Cancer cells must accumulate multiple mutations in the key cellular genes in order to attain the properties of autonomous replication and invasion. To become cancerous the cells require at least five successful gene mutations and with each mutation creating a cell increasingly well

adapted for autonomous growth in the host organism. The neoplastic cells acquire this by selecting the mutations that activates the oncogenes or inactivates the tumor suppressor genes (Bertram, 2001).

Cells can experience uncontrolled growth if there are mutations to DNA, and therefore, alterations to the genes involved in cell division. Four key types of gene are responsible for the cell division process: oncogenes tell cells when to divide, tumor suppressor genes tell cells when not to divide, suicide genes control apoptosis and tell the cell to kill itself if something goes wrong, and DNA-repair genes instruct a cell to repair damaged DNA. Cancer occurs when a cell's gene mutations make the cell unable to correct DNA damage and unable to commit suicide. Similarly, cancer is a result of mutations that inhibit oncogene and tumor suppressor gene function, leading to uncontrollable cell growth. Carcinogens are a class of substances that are directly responsible for damaging DNA, promoting or aiding cancer. Tobacco, asbestos, arsenic, radiation such as gamma and x-rays, the sun, and compounds in car exhaust fumes are all examples of carcinogens. When our bodies are exposed to carcinogens, free radicals are formed that try to steal electrons from other molecules in the body. Theses free radicals damage cells and affect their ability to function normally.

History

Cancer has been traced to occur even before the origin of ancestral species of man (Butler, 2004). The origin of the term lies in the observation that the blood vessels around malignant tumors looked like the claws of a crab to Hippocrates (ca. 460 BC –

ca. 370 BC), the father of modern medicine. He described several kinds of cancers, referring to them by the term *karkinos* (*carcinos*), the Greek word for crab or crayfish, as well as *carcinoma*. This comes from the appearance of the cut surface of a solid malignant tumour, with "the veins stretched on all sides as the animal the crab has its feet, whence it derives its name" (Moss and Ralph W, 2004).Humans have known cancer as a malignant disease as early as Ramayan and Egyptian (3000 to 1500 BC) periods. The Ramayana and Egyptian Papyrus even described the cure for cancer (Woelfer, 1881; Breasted, 1930; Ebbell, 1937). The Edwin Smith Surgical Papyrus, one of the eight Egyptian medical papyri, is believed to be the first document containing a record of human cancer. This text is believed to be written by Imhotep, the physician-architect around 3000–2500 BC, the pyramid age of Egypt. It recorded cancer as a disease without any treatment except palliative care (Breasted, 1984; Donegan, 2006; Sudhakar, 2009). The oldest evidence of cancer dates back to several million years ago and has been found in fossilized remains (bones) of a dinosaurs in Wyoming. The oldest specimens of cancer, a hominid malignant tumour (probably Burkitt's lymphoma) and bone cancer - were found in the remains of a body of either *Homo erectus* or an Australopithecus and in the remains of a female skull dating to the Bronze Age (1900-1600 B.C.). The earliest written records differentiating between benign and malignant cancers date back to ancient times (3000-1500 B.C., Mesopotamia and Egypt). Seven Egyptian Papyruses including the Edwin Smyth (2500 B.C.), Leyde (1500 B.C.), and George Ebers (1500 B.C.) described not only the symptoms but also the first primitive forms of treatment, i.e. the removal of a malignant tissue. The Hindu epic, the Ramayana

(500 B.C.), mentioned not only cancer cases but also the first medicines in the form of arsenic pastes, for treatment of cancerous growth. The earliest evidence of human cancer found till date is a metastatic carcinoma in a young man from ancient Nubia (presently an archaeological site of Amara West in modern Sudan, situated on the left bank of the Nile river) which dates back to 1200 BC (Binder *et al*., 2014). Evidence of sarcoma, a bone cancer as well as damage to the skull bone characteristics of head and neck cancer has been found in ancient mummies (American Cancer Society, 2010). In the 2nd century AD, the Greek physician Galen used *oncos* (Greek for swelling) to describe all tumours, reserving Hippocrates' term *carcinos* for malignant tumours. Galen also used the suffix *-oma* to indicate cancerous lesions. It is from Galen's usage that we derive the modern word oncology (Karpozilos and Pavlidis, 2004). The first cause of cancer was identified by British surgeon PercivallPott, who discovered in 1775 that cancer of the scrotum was a common disease among chimney sweeps. With the widespread use of the microscope in the 18th century, it was discovered that the 'cancer poison' eventually spreads from the primary tumor through the lymph nodes to other sites ("metastasis"). This view of the disease was first formulated by the English surgeon Campbell De Morgan between 1871 and 1874 (Grange JM *et al*., 2002). The use of surgery to treat cancer had poor results due to problems with hygiene. In the 19th century, asepsis improved surgical hygiene and as the survival statistics went up, surgical removal of the tumor became the primary treatment for cancer. With the exception of William Coley who in the late 19th century felt that the rate of cure after surgery had been higher before asepsis (and who injected bacteria into tumors with mixed results), cancer treatment

became dependent on the individual art of the surgeon at removing a tumor. The underlying cause of his results might be that infection stimulates the immune system to destroy left tumor cells.

Causes

Cancer is a class of diseases characterized by out-of-control cell growth**.** There are over 100 different types of cancer, and each is classified by the type of cell that is initially affected. There are many factors which contributes to the cause of cancer either directly or indirectly based on the environmental, lifestyle and behavioral exposures (Steward and Wild, 2014). The term "environmental", as used by cancer researchers, refers to everything outside the body that interacts with humans. In this sense, the environment is not limited to the biophysical environment (e.g. exposure to factors such as air pollution or sunlight, encountered outdoors or indoors, at home or in the workplace), but also includes lifestyle, economic and behavioral factors (Kravchenko *et al*., 2009). Common environmental factors that contribute to cancer death include tobacco (according to one estimate, accounting for 25–30% of deaths), obesity (30–35%), infections (15–20%), radiation (both ionizing and non-ionizing, up to 10%), lack of physical activity, and environmental pollutants (Anand *et al*.,2008).

Aging has been repeatedly and consistently regarded as an important aspect to consider when evaluating the risk factors for the development of particular cancers; aging is considered a risk factor and this is explained by the observation that many molecular and cellular changes are involved in the development of cancer, so it is very

likely that these changes accumulate during the aging process (that is, molecular and cellular changes collectively leading to cancer accumulate throughout the years), eventually manifesting themselves as cancer (WHO, 2014). Over 30% of cancers are potentially avoidable by reducing key risk factors, of which much the significant is tobacco use, which is the cause of about 22% of cancer deaths (WHO, 2014). Another 10% is due to obesity, a poor diet, lack of physical activity, and drinking alcohol (WHO, 2014). Other factors include certain infections, exposure to ionizing radiation, and environmental pollutants (Anand P *et al*., 2008). In the developing world nearly 20% of cancers are due to infections such as hepatitis B, hepatitis C, and human papillomavirus (WHO, 2014). These factors act, at least partly, by changing the genes of a cell (World Cancer Report, 2014). Typically many such genetic changes are required before cancer develops (World Cancer Report, 2014). Approximately 5–10% of cancers are due to genetic defects inherited from a person's parents (American Cancer Society, 2013).

Tobacco smoking is associated with many forms of cancer (Sasco *et al*., 2004) and causes 80% of lung cancer (Biesalski *et al*., 1998). Daily long-term vaping with a high voltage (5.0 V) electronic cigarette may generate formaldehyde-forming chemicals at a greater level than smoking, which was determined to be a lifetime cancer risk of approximately 5 to 15 times greater than smoking (Cooke *et al*., 2015). Smoking has been estimated to cause 90% of male and 75%–80% of female lung cancer deaths in the United States each year and is the major cause of lung cancer accounting for about 80 % of all lung cancers. Though the most addictive component, nicotine itself is not a carcinogen, about 70 carcinogens have been identified in tobacco smoke (Biesalski *et*

al., 1998; Hecht, 1999). Many mutagens are also carcinogens, but some carcinogens are not mutagens. Alcohol is an example of a chemical carcinogen that is not a mutagen (Seitz *et al*., 1998). In Western Europe 10% of cancers in males and 3% of cancers in females are attributed to alcohol (Schütze M *et al*., 2011).

Diet, physical inactivity, and obesity are related to approximately 30–35% of cancer deaths (Kushi *et al*., 2006). In the United States excess body weight is associated with the development of many types of cancer and is a factor in 14–20% of all cancer deaths (Kushi *et al*., 2006). Physical inactivity is believed to contribute to cancer risk not only through its effect on body weight but also through negative effects on immune system and endocrine system and has been linked with increased risk of cancer of the breast, colon, prostate, and pancreas and of melanoma (Booth *et al*., 2002; Kushi *et al*., 2006). In the United States, 14% of men and 20% of women cancer deaths are attributed to higher body mass index (Drewnowski and Popkin, 1997). More than half of the effect from diet is due to over nutrition rather than from eating too little healthy foods. Diet contributes to about 39-35 % of all cancers in the USA (Doll and Peto, 1981). High consumption of animal fat and red meat is linked with cancers such as breast, colon, and prostate (Armstrongand Doll, 1975). Folate deficiency, one of the most common vitamin deficiencies can cause chromosomal breaks due to the deficient methylation of uracil to thymine thereby increasing the risk of cancer (Ames and Gold, 1998). A diet rich in salt is linked to gastric cancer especially in Japan. The consumption of alcohol contributes to oral, esophageal, liver and breast cancer and chewing of betel nut is also linked to oral cancer. It has also been observed that immigrants often develop risk to cancer prevalent

in the country where they migrate suggesting the relationship between diet and cancer (Buell and Dunn, 1965; Park *et al*., 2008). .

Worldwide approximately 18% of cancer deaths are related to infectious diseases. This proportion varies in different regions of the world from a highest of 25% in Africa to less than 10% in the developed world (Anand *et al*., 2008). Viruses are the usual infectious agents that cause cancer but bacteria and parasites may also have an effect. A virus that can cause cancer is called an *oncovirus*. These include human papillomavirus (cervical carcinoma), Epstein–Barr virus (B-cell lymphoproliferative disease and nasopharyngeal carcinoma), Kaposi's sarcoma herpesvirus (Kaposi's sarcoma and primary effusion lymphomas), hepatitis B and hepatitis C viruses (hepatocellular carcinoma), and Human T-cell leukemia virus-1 (T-cell leukemias). Bacterial infection may also increase the risk of cancer, as seen in *Helicobacter pylori*-induced gastric carcinoma (Pagano JS *et al*., 2004). Parasitic infections strongly associated with cancer include *Schistosomahaematobium* (squamous cell carcinoma of the bladder) and the liver flukes, *Opisthorchisviverrini* and *Clonorchissinensis* (cholangiocarcinoma) (Samaras V *et al*., 2010).

Up to 10% of invasive cancers are related to radiation exposure, including both ionizing radiation and non-ionizing ultraviolet radiation (Anand *et al*., 2008). Additionally, the vast majority of non-invasive cancers are non-melanoma skin cancers caused by non-ionizing ultraviolet radiation. Sources of ionizing radiation include medical imaging, and radon gas. Radiation can cause cancer in most parts of the body, in

all animals, and at any age, although radiation-induced solid tumors usually take 10–15 years, and can take up to 40 years, to become clinically manifest, and radiation-induced leukemias typically require 2–10 years to appear (Little 2000). Children and adolescents are twice as likely to develop radiation-induced leukemia as adults; radiation exposure before birth has ten times the effect. Ionizing radiation is not a particularly strong mutagen. Residential exposure to radon gas, for example, has similar cancer risks as passive smoking. Low-dose exposures, such as living near a nuclear power plant, are generally believed to have no or very little effect on cancer development. Radiation is a more potent source of cancer when it is combined with other cancer-causing agents, such as radon gas exposure plus smoking tobacco (Little, 2000). Three independent stages appear to be involved in the creation of cancer with ionizing radiation: morphological changes to the cell, acquiring cellular immortality (losing normal, life-limiting cell regulatory processes), and adaptations that favor formation of a tumor. Even if the radiation does not strike the DNA directly, it triggers responses from cells that indirectly increase the likelihood of mutations. It is estimated that 0.4% of cancers in 2007 in the United States are due to CTs performed in the past and that this may increase to as high as 1.5–2% with rates of CT usage during this same time period (Brenner and Hall, 2007). Prolonged exposure to ultraviolet radiation from the sun can lead to melanoma and other skin malignancies. Clear evidence establishes ultraviolet radiation, especially the non-ionizing medium wave UVB, as the cause of most non-melanoma skin cancers, which are the most common forms of cancer in the world (Cleaver and Mitchell, 2000).

CHAPTER 1

The vast majority of cancers are non-hereditary ("sporadic cancers"). Hereditary cancers are primarily caused by an inherited genetic defect. Less than 0.3% of the population are carriers of a genetic mutation which has a large effect on cancer risk and these cause less than 3–10% of all cancer (Roukos, 2009). Some of these syndromes include: certain inherited mutations in the genes *BRCA1* and *BRCA2* with a more than 75% risk of breast cancer and ovarian cancer and hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome) which is present in about 3% of people with colorectal cancer (Cunningham *et al*., 2010) among others.

Some hormones play a role in the development of cancer by promoting cell proliferation (Henderson *et al*., 2000). Insulin-like growth factors and their binding proteins play a key role in cancer cell proliferation, differentiation and apoptosis, suggesting possible involvement in carcinogenesis (Rowlands *et al*., 2009). Hormones are important agents in sex-related cancers such as cancer of the breast, endometrium, prostate, ovary, and testis, and also of thyroid and bone. For example, the daughters of women who have breast cancer have significantly higher levels of estrogen and progesterone than the daughters of women without breast cancer. These higher hormone levels may explain why these women have higher risk of breast cancer, even in the absence of a breast-cancer gene. Similarly, men of African ancestry have significantly higher levels of testosterone than men of European ancestry, and have a correspondingly much higher level of prostate cancer. Men of Asian ancestry, with the lowest levels of testosterone-activating androstanediolglucuronide, have the lowest levels of prostate cancer. Other factors are also relevant: obese people have higher levels of some

hormones associated with cancer and a higher rate of those cancers. Women who take hormone replacement therapy have a higher risk of developing cancers associated with those hormones. On the other hand, people who exercise far more than average have lower levels of these hormones, and lower risk of cancer. Osteosarcoma may be promoted by growth hormones. Some treatments and prevention approaches leverage this cause by artificially reducing hormone levels, and thus discouraging hormone-sensitive cancers (Henderson BE *et al*., 2000).

Cancer treatment

Cancer can be treated by surgery, chemotherapy, radiation therapy, hormonal therapy, targeted therapy (including immunotherapy such as monoclonal antibody therapy) and synthetic lethality. The treatment of cancer has undergone evolutionary changes as understanding of the underlying biological processes has increased with time. Tumor removal surgeries have been documented in ancient Egypt, hormone therapy and radiation therapy were developed in the late 19th century. Chemotherapy, immunotherapy and newer targeted therapies are products of the 20th century.

Surgery

Surgery is the oldest type of cancer treatment and is still in use in today's world and plays an important role in the removal of solid tumors. Surgery can be used as a cure if the cancer is detected in the early stages. It is known to be most effective and gives higher success rate for treatment of cancer than the other form of treatment when used alone (Harvey, 1974; Caley and Jones, 2012). Surgery is most effective in the treatment

of localized primary tumors and associated regional lymphatics and it is achieved by combining surgical procedures that attempt to encompass gross and microscopic tumor in all contiguous and adjacent anatomic locations. Some traces of the cancer cells may be missed during surgery and by removing the tissue extensively the surrounding normal tissue may become vulnerable and could impair the functioning. Moreover, it cannot be used for the treatment of later stages of cancer that has already metastasized (Greene, 2002).

Radiation Therapy

Radiation therapy (also called radiotherapy, X-ray therapy, or irradiation) is the use of ionizing radiation to kill cancer cells and shrink tumors. Radiation therapy can be administered externally via external beam radiotherapy (EBRT) or internally via brachytherapy. The effects of radiation therapy are localized and confined to the region being treated. Radiation therapy injures or destroys cells in the area being treated (the "target tissue") by damaging their genetic material, making it impossible for these cells to continue to grow and divide. Although radiation damages both cancer cells and normal cells, most normal cells can recover from the effects of radiation and function properly. The goal of radiation therapy is to damage as many cancer cells as possible, while limiting harm to nearby healthy tissue. Hence, it is given in many fractions, allowing healthy tissue to recover between fractions. The use of radiation as cancer treatment became common in the 1950s when Cobalt-60 gamma radiation became available followed by particle accelerators in the 1970s (Cuttler and Pollycove, 2003). Radiotherapy may be used either alone or in combination with surgery or chemotherapy

to cure cancer, where it may eliminate the cancer or prevent the recurrence of cancer. It may also be given with palliative intent where the purpose is not to cure but to relieve the symptoms caused by cancer. The radiation given before surgery to shrink the tumor is called neoadjuvant therapy whereas the radiation used after surgery to destroy the microscopic tumor cells after surgery is called adjuvant therapy.

The ionizing radiation kills or cause genetic alterations in the cells it passes through by depositing energy in the form of ions which damages DNA causing single or double or strand breaks (Lomax *et al*., 2013). This DNA damage occurs not only in tumor cells but also in the normal cells which are adjacent or nearby to the tumor, which changes the fidelity of genome causing cancer recurrence. However, cancer cells are less efficient than normal cells in repairing damage resulting in differential cancer killing (Begg *et al*., 2011). A major limitation of radiotherapy is the tumor cells that are in a low-oxygen state called as hypoxia which are 2 to 3 time more resistant to radiation damage as compared to those growing in a normal oxygenated environment (Harrison *et al*., 2002). Usually combination therapy is incorporated in such situations.

Chemotherapy

Chemotherapy is the treatment of cancer with drugs ("anticancer drugs") that can destroy cancer cells. In current usage, the term "chemotherapy" usually refers to *cytotoxic* drugs which affect rapidly dividing cells in general, in contrast with *targeted therapy*. Chemotherapy drugs interfere with cell division in various possible ways, e.g. with the duplication of DNA or the separation of newly formed chromosomes. Most forms of chemotherapy target all rapidly dividing cells and are not specific to cancer

cells, although some degree of specificity may come from the inability of many cancer cells to repair DNA damage, while normal cells generally can. Hence, chemotherapy has the potential to harm healthy tissue, especially those tissues that have a high replacement rate (e.g. intestinal lining). These cells usually repair themselves after chemotherapy. Some drugs work better together than alone, two or more drugs are often given at the same time. This is called "combination chemotherapy"; most chemotherapy regimens are given in a combination (Takimoto and Calvo, 2008).

There are more than 100 different types of chemotherapeutic drugs for the treatment of different types of cancers, which are used either alone or in combination. The alkylating agents directly damage the DNA of cancer cells and prevent them from reproducing. They can be used to treat different types of cancers since they kill the neoplastic cells in any stages of the cell cycle. Nitrogen mustards, nitrosoureas, alkyl sulfonates, triazines and ethyleneamines are different types of alkylating agents (Colvin, 2003).Unlike the alkylating agents, the antimetabolites work only during the S-phase of the cell cycle and drive the cell to programmed cell death. They hinder the synthesis of DNA or RNA by inhibiting the enzymes necessary for DNA synthesis thereby preventing mitosis and can also incorporate themselves into the DNA since many of them have structures similar to the nucleotides. The anti-folates, fluoropyrimidines, deoxynucleoside analogues and thiopurines are the different types of anti-metabolites, which are used in clinics for the treatment of cancer (Lind, 2008; Parker, 2009).

Vincristine and vinblastine isolated from *Catharanthus roseus* are examples of vinca alkaloids whereas paclitaxel extracted from *Taxusbrevi folia* is an example of

taxanes (Lind, 2008; Yue *et al*., 2010; Liu *et al.,* 2007). Another group consists of cytotoxic antibiotics, which interrupt cell division by intercalating into the DNA. The doxorubicin an anthracycline and bleomycin are the subgroups in the antibiotic category. They are used in the treatment of different types of neoplasia like breast, ovary, bladder and lung cancers, and lymphomas and sarcomas (Chabner and Longo, 2001).

Chemotherapy drugs are given repeatedly at a regular time intervals known as treatment cycles. Each cycle kills a fraction of cells, and not a constant number of cells. Since normal cells have more capacity to repair as compared to tumor cells, the repeated cycles allow normal cells to repair and repopulate while tumor cells constantly decrease in numbers (Caley and Jones, 2012). A detectable cancer usually has gone through over 30 doublings and contains 10^8-10^9 cells (Price *et al*., 2008). Therefore, even though not detected after treatment there is still considerable number of cells which can cause relapse of the disease (Caley and Jones, 2012).

Chemotherapy also has its own limitations as it does not specifically target tumor cells. The major drawback of cancer chemotherapy is that the drugs destroy even the body's immune cells which has to be replaced by bone marrow transplantation and highly toxic to normal cells (Chabner, 2001). The chemotherapeutic agents hamper cell division or inhibit enzymes involved in DNA replication or metabolism, they also damage the normal dividing cells especially the rapidly regenerating tissues, such as those of the bone marrow, gut mucosa and hair follicles (Wu *et al*., 2008). Further, it can also lead to the development of drug resistant cells and many of the drugs that kill tumors can cause mutations that transform normal cells into cancer cell (Aqeilan *et al*.,

2009). Another drawback of chemotherapy is that the non-homogenous cancer stem cells are not affected by chemotherapy and therefore cannot be eliminated even if all the cancer cells die. These cancer stem cells thereby can cause cancer again (Cetin and Topcul, 2012). Combination chemotherapy is often used to provide maximum cell kill at lower toxicity to the host and to prevent the development resistance (Page and Takimoto, 2004).

AIM OF THE STUDY

From the above it is clear that new paradigms are required to treat cancer that do not lead to adverse side effects of modern chemotherapy. Plants have been the major source for several drugs and it is well known that 75% of the modern chemotherapeutic drugs have their origin in plants or natural products (Cragg and Newman, 2013; Harvey *et al*., 2015). Several phytoceuticals have been screened for their anticancer activities earlier. Many plants have been found to possess anticancer activity in different preclinical systems (Jagetia *et al*., 1998; Jagetia and Baliga, 2005; Jagetia *et al*., 2005, Jagetia and Venkatesha, 2012; Rosangkima and Jagetia, 2015). Extracts of some plants have also been reported to exert anticancer activity (Solowey *et al*., 2014). Therefore plants and natural products still provide a major avenue for screening of new drug entities and to develop new non-toxic molecules for cancer treatment.

*Colocasia gigantea (*also called giant elephant ear or Indian taro) is a 1.5-3 m tall herb with a large, fibrous, inedible corm, producing at its apex a whorl of large leaves. The leaf stalks are used as a vegetable in some areas in South East Asia and Japan. *C.gigantea* grows commonly in Thailand and other Southeast Asian countries (Manner,

2011). In the Pacific islands, the tubers are cooked and eaten as a starch (Manner, 2011). In India and Bangladesh, the tubers are used as a main ingredient in curries and stews (Kay, 1987). In Thailand, *C. gigantea* is considered as a minor food crop and is mainly utilized as a stem vegetable. In Thai traditional medicine, *C. gigantea* tuber is heated over a fire. It is used to reduce "internal heat" (fever) and also for the treatment of drowsiness. Fresh tuber has been shown to ameliorate stomach problems, combat infection, and accelerate the healing of wounds. In the northern region of Thailand, fresh or dried tuber is used for the treatment of phlegm by mixing it with honey.

Therefore, the present study was designed to study anticancer activity of *Colocasia gigantea* extract/s *in vitro* and *in vivo* by carrying out the following investigations:

1. Phytochemical analysis.
2. Evaluate antioxidant activity of different extracts of *Colocasia gigantea.*
3. Study of the anticancer activity of *Colocasia gigantea* in Dalton lymphoma tumour bearing mice.
4. Study the cytotxic effect of *Colocasia gigantea* in vitro.

CHAPTER 1

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CHAPTER 2

PHYTOCHEMICAL SCREENING OF VARIOUS EXTRACTS OF *COLOCASIA GIGANTEA* (BLUME) HOOK. *f. IN VITRO*

CHAPTER 2

Abstract

The non-infected rhizomes of Colocasia gigantea were collected, powdered and sequentially extracted with petroleum ether, chloroform, ethanol and distilled water using a Soxhlet apparatus. All the extracts except petroleum ether were cooled, solidified and stored at -70^{o}C until further use. The different extracts were analyzed for presence of various phytochemicals using standard protocols. The TLC profiles of various extracts were obtained using different solvent systems. The Colocasia gigantea showed the presence of alkaloids, cardiac glycosides, phytosterols, saponins, tannins and flavonoids. The TLC study also showed the presence of various components as indicated by the different Rf values in different solvent systems.

CHAPTER 2

1. INTRODUCTION

The plant synthesize different chemical for various purposes and plant-derived substances have recently become of great interest owing to their versatile applications. Medicinal plants have been the richest bio resource of drugs of traditional systems of medicine, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for modern synthetic drugs. The application of plants as medicines is as old as human civilization. Use of plants as medicine has been recorded throughout the human history in different parts of the world. The written records in the old Hindu scripture, the Rig Veda, dating back to 3500 B.C. to 1800 B.C. The Atharvaveda (1200 BC), Charak Samhita and Shusrut Samhita (1000-500 BC) are the main classical texts that give a detailed account of more than 700 herbs (Dash and Sharma, 2001). The oldest known written medical prescription dates back to four thousand year in Sumerian clay tablets that has listed the remedies for various illnesses where plants were given for pain relief, turmeric was applied to trigger blood clotting, roots of endive plant to cure gall bladder disorders, and raw garlic to treat circulatory disorders (Kong *et al.,* 2003). The popularity of plant based medicines for healthcare has been are not only due to low economical costs but also due to their greater safety than the modern synthetic drugs. This is reflected by the fact that more than 80% of the world's inhabitants use plants or natural products for their healthcare (WHO, 2010).

The other factor that has contributed to the increasing popularity of herbal medicines worldwide is the growing recognition that natural products are less toxic and do not induce any known adverse side effects or they are less toxic than the synthetic drugs due to their biologic origin (Jagetia, 2017). The plants and other

natural products have served as a main source to isolate or synthesize several modern drugs, which were initially extracted from natural sources before their actual synthesis was carried out. The several antibacterial, antimycotic and anticancer drug which are sold in the markets were initially isolated from medicinal plants or other natural sources (Cragg and Newman, 2013; Kinghorn, 2016; Newman and Cragg, 2016). The isolation and characterization of pharmacologically active compounds from medicinal plants still continue today in the hope to find better non-toxic medicines as a cure for various human health disorders.

The phytochemicals synthesized by plants can be mainly grouped into primary and secondary metabolites (Irchhaiya *et al.,* 2015). The primary metabolites include phytosterols, acyl lipids, amino acids and organic acids that have shared biological function across all plant species (Waterman, 1992) and are mainly responsible for growth, development and other metabolic activities essential for the survival of plants (Croteau *et al.,* 2000). The metabolism of primary metabolites generates secondary metabolites, which are not involved in any of the vital activities of plants required for their sustenance (Irchhaiya *et al.,* 2015). The plants usually synthesize these phytochemicals in specialized cells during particular developmental phase making their extraction and purification difficult (Shula *et al.,* 2009). The secondary metabolites are useful to defend plants against pathogenic and insect attack, herbivory, pollination and protect against environemtal stress (Li *et al.,* 1993; Stamp, 2003; Qin *et al.,* 2011; Samuni-Blank *et al.,* 2012). These phytochemicals produce various biological activities in humans, and this has been the reason that plants have been used to treat several ailments in traditional medicine since the time immemorial. It is also known that almost 70% of the modern medicines have a direct

or indirect origin in plants (Newmann and Cragg, 2014). The various phytochemicals synthesized by plants as secondary metabolites have been found to exert various physiological effects in mammals including humans and hence they are also called the active principle of that plant (Shula *et al.,* 2009).

Colocasia gigantea (family: Araceae) is an erect, evergreen, perennial. Stemless plant that produces a cluster of large leaves from an underground rhizome. It grows upto a height of 4 metres (Manner, 2011). The plant is harvested from the wild for local use and rhizomes are cooked and eaten as a vegetable or eaten raw. Leaves are also eaten in some parts of India. It is often grown as an ornamental plant in warmer climates. *Colocasia gigantea* is also occasionally cultivated as a food plant in Southeast Asia. The leaf stems are cooked and eaten as vegetables in India, Japan and Bangladesh (Kay, 1987). The fruit is an oblongoid berry, about 10 mm long and smell like *Alpiniamalaccenis* (Burm.f.) Roscoe. The fruits are used as a flavoring agent. The corms of *Colocasia gigantea* carry high- quality phytonutrients comprising of dietary fibre, and antioxidants in addition to moderate properties of minerals and vitamins. Fresh tubers of *Colocasia gigantea* have been shown to ameliorate stomach problems, combat infection, and accelerate the healing of wounds. In the northern region of Thailand, fresh or dried tubersare used for the treatment of phlegm by mixing it with honey (Essence of the Agriculture, Songkla University; 2006). Since *Colocasia gigantea* is part of diet, the present study was undertaken to investigate the phytochemical constituents present in its rhizomes.

Colocasia gigantea (family: Araceae) also called Giant elephant ear or Indian taro is grows up to a height of 1.5-3 m with a whorl of large leaves at its apex. It bears a large, fibrous, and inedible corm. *C. gigantea* grows commonly in India,

Thailand and other Southeast Asian countries. The leaf stalks are used as a vegetable in some areas in, India, South East Asia and Japan. In India C the tubers are cooked and used as a vegetable (Kay, 1987). In Thailand, *C. gigantea* is considered as a minor food crop and is mainly utilized as a stem vegetable. *C. gigantea*'s stalks are often used for making homemade Thai food called "Bon curry". In the Pacific islands, the tubers are cooked and eaten as a starch (Manner, 2011). In Thai traditional medicine, *C. gigantea* tuber is heated over a fire and is used to reduce "internal heat" (fever) and also for the treatment of drowsiness. The fresh or dried tubers mixed with honey are used in the treatment of phlegm in northern Thailand. Fresh tubers have been shown to ameliorate stomach problems, combat infection, and accelerate the healing of wounds. Therefore, the present study was undertaken to investigate the phytochemicals properties present in *Colocasia gigantea.*

2. MATERIALS AND METHODS

2.1. Chemicals

Potassium iodide, bismuth nitrate, sulphuric acid, ferric chloride, hydrochloric acid, aluminium chloride, ammonium hydroxide, glacial acetic acid, chloroform, ethanol, methanol, n-butanol, ethyl acetate, sodium chloride, sulphuric acid, olive oil and Whatman filter paler were procured from SD fine Chemical Ltd., Mumbai, India. The TLC plates were commercially procured from Merck India, Mumbai.

2.2. Collection and Extraction

The plant was identified by PG Department of Botany, DM college, Imphal-West, Manipur, India and further authenticated by Botanical Survey of India, Shillong, Meghalaya, India. The non-infected and matured rhizomes of *Colocasia*

gigantea were collected from Manipur. The rhizomes of *Colocasia gigantea* were washed thoroughly with clean water, their skins were removed and cut in to small pieces so as to facilitate drying. The chopped pieces of died rhizomes were spread into the stainless steel trays and shade dried at room temperature in dark, clean and hygienic conditions so as to block the entry of insects, animals, and extraneous terrestrial materials. The dried rhizomes were kept in a grinder and powdered at room temperature. A sample of 100 g of rhizome powder was extracted sequentially with chloroform, ethanol and water in a Soxhlet apparatus (Suffness and Dorous, 1979). The resultant extracts were concentrated to dryness under reduced pressure and stored at -80 until further use. The chloroform, ethanol and water extracts of *Colocasia gigantea* will be called as CGC, CGE and CGA henceforth.

2.3. Phytochemical Screening

The screening of different extracts of *C. gigantea* was carried out for the presence of various phytochemicals using standard procedures described below:-

2.3.1. Alkaloids

The presence of alkaloids in *C. gigantea* was confirmed by employing the Dragendorff's test of different extracts of *C.gigantea* (0.1g) were mixed with 0.5 ml of Dragendorff's reagent. The development of reddish brown precipitate indicates the presence of alkaloids (Harborne, 1998; Kokate *et al.,* 2006; Doughari, 2012).

2.3.2. Carbohydrates

The presence of carbohydrates was detected by performing the Benedict's test. 0.1g of each extracts was dissolved individually in 5 ml distilled water and filtered. The filtrates of the different extracts were treated with Benedict's reagent

individually and were heated gently. Orange precipitate indicates the presence of reducing sugars. (Harborne, 1998; Kokate *et al.,* 2006; Doughari, 2012).

2.3.3. Tannins

The presence of tannins was determined by Ferric chloride test. About 0.5 g of dried powdered extracts were boiled in 20 ml of water in a test tube and filtered. To the filtrate a few drops of 0.1% ferric chloride was added that led to the formation of brownish green or a blue-black colour (Harborne, 1998; Kokate *et al.,* 2006; Doughari, 2012).

2.3.4. Steroids

The presence of steroids in various extracts of *C. gigantea* was determined by Salkowski's test. Briefly, 0.1 g of various extracts of *C. gigantea* were dissolved in different solvents and mixed with a few drops of concentrated sulphuric acid. The development of red colour at lower layer indicates the presence of steroids, whereas the formation of yellow colour indicates the presence of triterpenoids (Harborne, 1998; Kokate *et al.,* 2006; Doughari, 2012).

2.3.5. Flavanoids

The flavonoids were qualitatively estimated using alkaline reagent test, where 0.1 g of each extract of *C. gigantea* was dissolved in appropriate solvents and mixed with a few drops of sodium hydroxide solution. The formation of intense yellow colour, which turns colourless after addition of a few drops of dilute acid indicates the presence of flavonoids (Sofowara, 1993; Harborne, 1998; Kokate *et al.,* 2006; Doughari, 2012).

2.3.6. Saponins

Usually 2 g of powdered sample was boiled with 20 ml of distilled water in a water bath for 10 minutes and filtered while hot and cooled before conducting the following tests:

Frothing: 3 ml of filtrate was diluted up to 10 ml with distilled water and shaken vigorously for 2 minutes. The formation of a fairly stable froth indicates the presence of saponins.

Emulsification: Three drops of olive oil were added to the solution obtained by diluting 3 ml filtrate to 10 ml distilled water and shaken vigorously for a few minutes. The formation of a fairly stable emulsion indicates the presence of saponins (Trease and Evans 1989; Harborne, 1998; Doughari, 2012).

2.3.7. Cardiac glycosides (Keller-Killani test)

Usually 5 ml of each extracts was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution with an under laying of 1ml of concentrated sulphuric acid. The appearance of brown ring at the interface indicates the presence of deoxysugar, which is a characteristic of cardenolides (Harborne, 1998; Doughari, 2012).

2.4. Quantitative determination of the phytochemicals

2.4.1. Alkaloids

10 g of rhizome powder of *Colocasia gigantea* were weighed and transferred into the 250 ml beaker followed by the addition of 200 ml of 10% acetic acid in ethanol. The flask was covered and allowed to stand for 4 hours and filtered. The

filtrate was concentrated on a water bath to one-quarter of the original volume followed by the drop wise addition of concentrated ammonium hydroxide until complete precipitation. The whole solution was allowed to settle and the precipitate was collected and washed with dilute ammonium hydroxide and filtered. The residue containing the alkaloids, was dried, weighed and percentage of alkaloids was calculated (Harborne, 1998).

2.4.2. Flavonoids

10 g of powdered rhizomes of *Colocasia gigantea* were extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature. The whole solution was filtered through Whatman filter paper No. 42 (125 mm). The filtrate containing flavonoids was transferred into a crucible and evaporated to dryness over a water bath and weighed (Hagerman et al., 2000).

2.5. Ash Content

The crude rhizomes powder of *Colocasia gigantea* were weighed and heated at 500-600ºC in a Nabertherm muffle furnace until it became white, indicating the absence of carbon. The crucible was cooled and weighed. The percentage of total ash content was calculated according to the following formula.

Total ash content = Pw-Fw/W x 100

Where Pw= Preweighed crucible

Fw= Final weight of the crucible containing ash

W= Total weight of powdered plant material

2.6. Determination of moisture content:

Determination of the amount of volatile matter (i.e., water drying off from the sample) in the sample is a measure of loss after drying of substances appearing to contain water as the only volatile constituent. The powdered rhizome of *Colocasia gigantea* was accurately weighed, placed (without preliminary drying) in a tared evaporating dish, dried at 105˚C for 5 hours, and weighed once again. The percentage moisture content was calculated with reference to the initial weight using the following formula:-

$$\text{Moisture content} = \frac{Pw-Fw}{W} \times 100$$

Where Pw = Preweighed sample

Fw = Final weight of the dried sample

W = Total weight of the sample

2.7. Thin Layer Chromatography

Thin Layer Chromatography (TLC) is a simple and rapid technique used to determine the number of components present in any pharmacological agent and helps in finding a suitable solvent for separating the components by column chromatography as well as for monitoring reactions' progress.TLC was performed on the different extracts of *Colocasia gigantea* to allow the separation of various phytochemical compounds present in them . The extracts were applied onto activated (100°C for 30 minutes) and cooled silica gel TLC plates (60GF254, 20x20 cm; 0.2-0.3 mm thick, Merck India, Mumbai, India) (Harborne, 1973).The TLC plates were placed into different closed chambers containing various solvent systems in order to identify the varied compounds present in the different extracts. The solvent system

consisted of chloroform: methanol (5:5; 6:4; 7:3) for chloroform extract and chloroform: methanol (4:6; 5:5; 6:4; 7:3) for ethanol extract respectively. The resultant spots were visualizeded under visible as well as ultra-violet light. The value of the Retention factor (Rf) was calculated using the formula:-

Rf = Distance travelled by solute/ Distance travelled by solvent

3. RESULTS

The results of phytochemical analyses of *Colocasia gigantea* are presented in Table 1-4 and Figures 1-5.

Alkaloids

The presence of alkaloids was confirmed with Dragendorff's reagent in ethanol extract, which indicated the presence of reddish brown precipitate. However, alkaloids were completely absent in both the chloroform and aqueous extracts (Table 1).

Carbohydrates

The presence of carbohydrates was confirmed by the Benedict's Test only in ethanol extract and is indicated by the presence of orange precipitate indicating the presence of reducing sugars. The carbohydrates were absent in both the chloroform and aqueous extracts (Table 1).

Tannins

Tannins were present in both the chloroform and aqueous extracts which was indicated by presence of blue-black precipitate (Table 1). However, ethanol extract did not show positive result for the presence of tannins (Table 1).

Phytosterols

The development of red colour at lower layer indicated the presence of steroids in both the chloroform and ethanolic extracts whereas phytosterols were completely absent in aqueous extract (Table 1).

Flavanoids

The analysis of flavonoids in all the three extract showed their presence only in chloroform extract of *Colocasia gigantea* as indicated by the formation of intense yellow colour (Table 1). However, both the ethanol and aqueous extracts were negative for the flavonoids (Table 1).

Saponins

The formation of emulsion in the ethanol extract indicates the presence of saponins which were complexly absent in both the chloroform and aqueous extracts (Table 1).

Cardiac Glycosides

The cardiac glycosides were present in the chloroform extract as indicated by the presence of brown ring at the interface (Table1). However cardiac glycosides were absent in both the ethanol and aqueous extracts (Table 1).

Ash Content:

The ash content of the crude rhizome powder was found to be 20.5% (Table 2).

Moisture Content:

The estimation of moisture content in the crude *Colocasia gigantea* rhizome powder showed moisture content of 31.8 % (Table 2).

CHAPTER 2

Extractive Yield

The extraction of 5kg *Colocasia gigantea* rhizomes yielded 491g of chloroform extract, 501g of ethanol extract and 513g of aqueous extract, respectively (Table 3).

Quantification of phytochemicals

The quantitative estimation of alkaloids and flavonoids revealed that the ethanol extract contained 6.2 % alkaloids only whereas the chloroform extract was found to contain 46% flavonoids only (Table 4).

TLC Analysis

The evaluation of various extracts of *Colocasia gigantea* showed presence of different components as indicated by a varying number of spots on a TLC plates. The TLC profiles are depicted in (Table 5 and 6, Figures 2 to 5).

DISCUSSION

The phytochemical analysis of plants is an integral part of new drug development and discovery and it also plays an important role in the combinatorial chemistry for new drug discovery. The presence of numerous distinct phytochemicals or pharmacophores makes plants as an important and renewable source of medicines that have been and are used to cure various human ailments. Plants are considered non-toxic and safer than other exotic pure chemicals. This may be due to the fact that phytoceuticals origin is biological and also they have been experimented since the advent of human history (Jagetia and Venkatesha, 2005; Shantabi *et al.,* 2014).Therefore the present study was designed to evaluate the presence of different phytochemicals in *Colocasia gigantea* using standard protocols.

CHAPTER 2

The alkaloids are organic bases and plants have been reported to produce more than 20000 diverse alkaloids (Matsuura and Fett-Neto, 2015).The alkaloids have played a key role in the treatment of human diseases since the advent of human history. The several plant derived alkaloids including vinca alkaloids, taxens, podophyllotoxins, camtothecins etc. (Nicolaou *et al*., 1994; Moudi *et al.,* 2013) have formed the basis of cancer chemotherapy in the modern medicine. Some other alkaloids such as morphine and cocaine have been or are still used as local anaesthetic, stimulants and pain relievers (Rothman *et al.,* 2001; Karamese *et al.*, 2015). The plant alkaloids like caffeine nicotine, and antimalarial drug quinine also act as stimulants (Dani and Bertrand, 2007; Thompson and Lummis, 2008; Prasad, 2011).

Flavonoids are important colourful polyphenolic biomolecules which are synthesized by most plants for various purposes and more than 6000 different flavonoids have been identified in different plants. The flavonoids are mainly responsible for the beautiful colours of flowers along with anthocyanins (Iwashina, 2015). The flavonoids serve as co-pigment/s contributing to variation in the flower colours and fruits. The flavonoids are secondary metabolites and have important functions in the plants that include stimulation, protection, flavouring, pigmentation and in plant-microorganism communication in the plants (Ghasemzadeh and Ghasemzadeh, 2011). They are essential in pollination and seed dispersal and protect plants from the UV light and other stresses and help in the development (Petrussa *et al.,* 2013; Brunetti *et al.,* 2016). The flavonoids also protect plants and humans against the oxygen-induced oxidative stress (Brunetti *et al.,* 2016). The flavonoid have been reported to trigger several beneficial effect in human as they have been

reported to be antiallergic, anticancer, antibacterial, anticatatactogenic, antidiabetic, cardioprotective, hepatoprotective, antiosteoporotic, antiinflammatory, and antiviral (Hegarty *et al.,* 2000; Cushnie and Lamb, 2005; Chahar *et al.,* 2011; Kumar and Pandey, 2013; Tanaka, 2013; Ivey *et al.,* 2015).

Cardiac glycosides are another class of phytochemicals syntheisized as secondary metabolites by plants and they have also been reported to possess numerous medicinal properties. The first medical application of cardiac glycosides has been reported 1500 years back. They have been used applied on arrows as poisons of killing. They also found their application as emetics, diuretics, cardiotonics and to induce abortions (Robert *et al.,* 2008). The cardiac glycosides were found to exert anticancer effects as early as 1967 The other preclinical studies reported that cardiac glycosides are cytotoxic to breast, lung, prostate, and pancreatic cancers, skin melanomas, leukaemia, neuroblastoma and renal adenocarcinoma (Felth *et al.,* 2009). the cardiac glycosides have been found to inhibit the carcinogenesis in preclinical models (Prassas and Diamandis, 2008). The cardiac glycosides have been indicated in the treatment of cardiovascular diseases (Menger *et al.,* 2012).

The phytochemicals that form froth in water are known as saponins which are triterpinoids synthesized by plants. The saponins are usually aglycones, designated genins or sapogenins, which covalently bound to one or more sugar moieties. The saponins helps plants to fight against pathogen attack and animals that eat plants that is herbivores. The sapnoins act as allelopathic fungicidal, insecticidal, and molluscicidal (Aladesanmi, 2007; Sung *et al.,* 2008; Nielsen *et al.,* 2010). Apart from

these they possess various other activities and they are anticarcinogenic, antiprotozoal, antifungal, immunoadjuavant, antiviral, antioxidant, immunostimulatory, hypoglycemic and membrane permeabilizing (*Khatuntseva et al.,* 2012; Khan *et al.,* 2012). Saponins inhibit angiogenesis and possess anticancer activity. They have been reported to block the progressing of the cell cycle and induce apoptosis in cultured cell lines (Man *et al.,* 2010).

The another class of polyphenolic compounds synthesized by plants as secondary metabolites are tannins, which are structurally complex phytochemicals, (Khanbabaee and van Ree, 2001; Lu *et al.,* 2012). The tannins are good weapons to protect plants against herbivory and attacks by insects as they can induce toxicity (Robbins *et al.,* 1987; Frutos *et al.,* 2004) for the tannins also play a crucial role in protecting plants against the microorganisms (Barbehenn and Constabel, 2011). Tannins have dual action they act as antioxidants and they become prooxidants oxygen atmosphere (Robbins *et al.,* 1987). The tannins possess numerous activities like antibacterial, anti-inflammatory, antitumour, astringent, antiulcerogenic, antiviral, and antithrombogenic (Takechi and Tanaka, 1987; Banso and Adeyemo, 2007; Clinton, 2009; Ashok and Upadhyaya, 2012).

Phytochemical analysis and TLC profiling of various extracts of *Colocasia gigantea* revealed that it synthesizes alkaloids, cardiac glycosides, flavonoids, phytosterols, saponins, and tannins. The medicinal activities of this plant may be attributed to one or more of these phytochemicals or also it may be also due to the combined activity of all these chemicals.

CHAPTER 2

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Table 1. Phytochemical analysis of different extracts of *Colocasia gigantea* rhizomes.

TESTS	Chloroform Extract	Ethanol Extract	Aqueous Extract
Alkaloids	-	+	-
Carbohydrates	-	+	-
Tannins	+	-	+
Phytosteroids	+	+	-
Flavanoids	+	-	-
Saponins	-	+	-
Cardiac Glycosides	+	-	-

Table 2. Physicochemical parameters of dried rhizomes powder of *Colocasia gigantea*.

Moisture Content	Ash Content
31.8 %	20.5 %

Table 3. Yield of various extracts of dried rhizomes powder of *Colocasia gigantea.*

Rhizomes powder	Chloroform Extract	Ethanol Extract	Aqueous Extract
5kg	491g	501g	513g

Table 4. Quantitative determination of the chemical constituent of *Colocasia gigantea*.

Phytochemicals	Initial Weight (g)	Final Weight (mg)	Content (%)
Alkaloids	10	0.62	6.2
Flavonoids	10	4.6	46

Table 5: Rf value of chloroform extract using TLC plates.

Solvent system	Rf Value
Chloroform: Methanol (5:5)	0.18
Chloroform: Methanol (6:4)	0.15
Chloroform: Methanol (7:3)	0.17

Table 6: Rf value of ethanol extract using TLC plates.

Solvent system	Rf Value
Chloroform: Methanol (4:6)	0.27
Chloroform: Methanol (5:5)	0.26
Chloroform: Methanol (6:4)	0.23
Chloroform: Methanol (7:3)	0.25

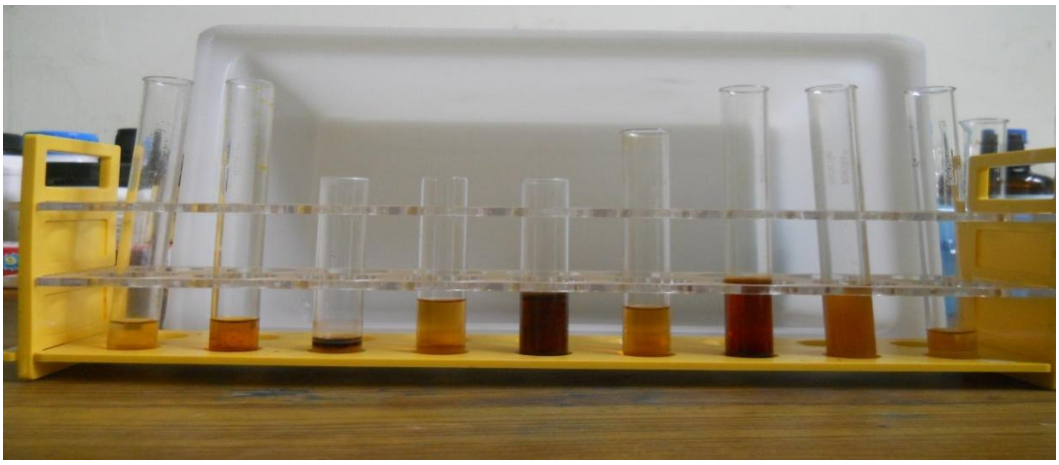

Figure 1: Phytochemical profile of various extracts of *Colocasia gigantea* rhizomes.

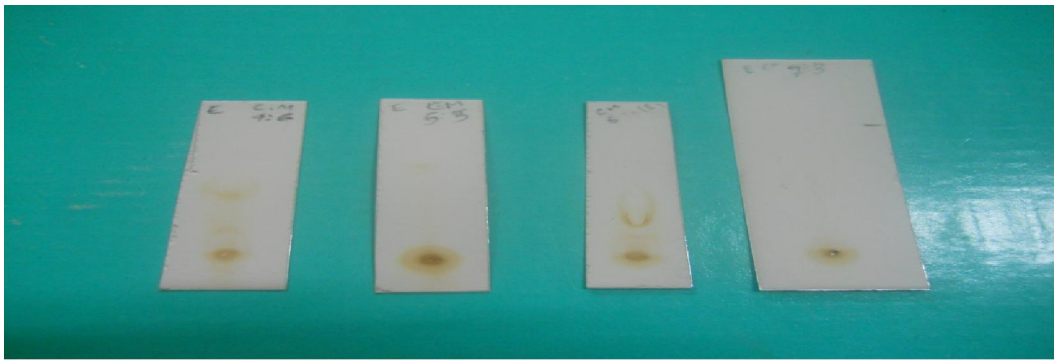

Figure 2: TLC Profile of ethanol extract of *Colocasia gigantea* using solvent system chloroform: methanol in the ratio of 4:6, 5:5, 6:4 and 7:3

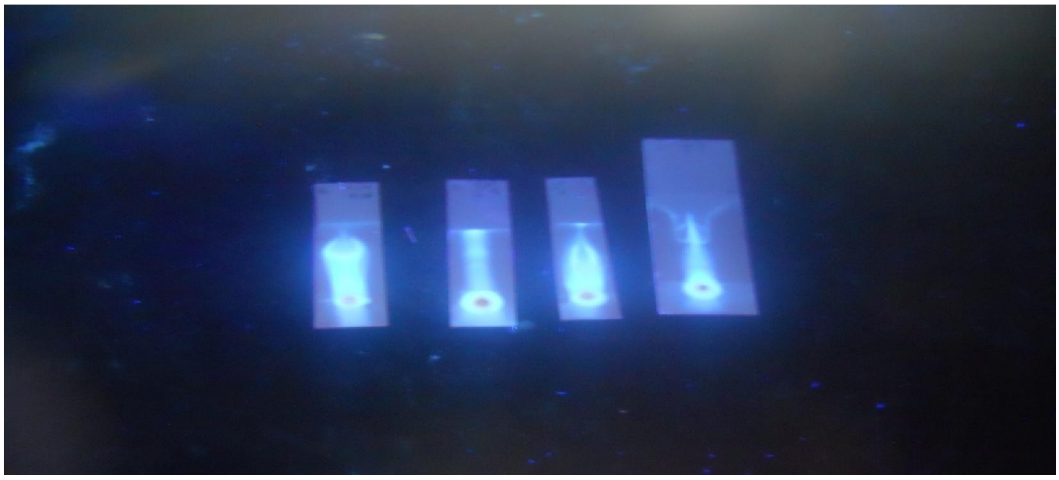

Figure 3: TLC Profile of ethanol extract of *Colocasia gigantea* under UV 365 nm using solvent system chloroform: methanol in the ratio of 4:6, 5:5, 6:4 and 7:3

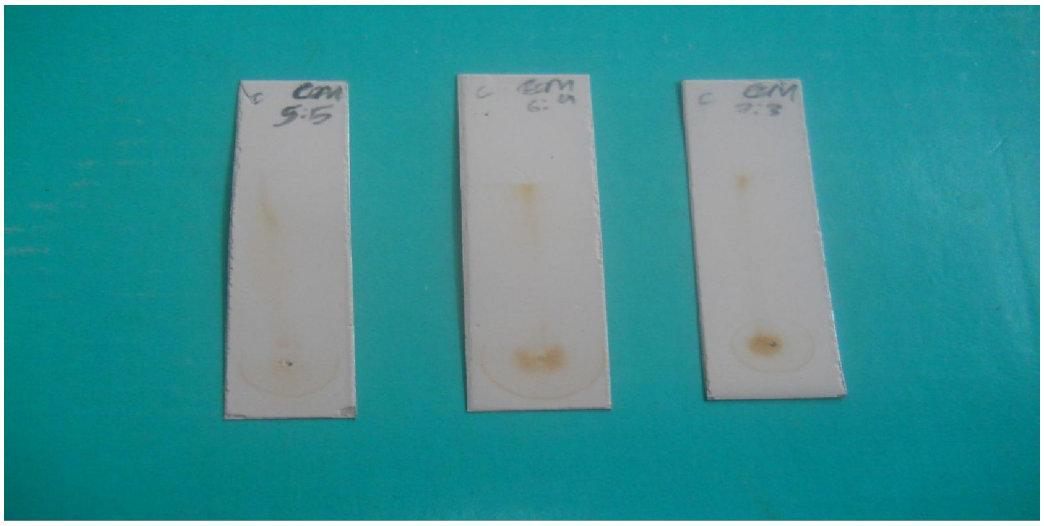

Figure 4: TLC Profile of chloroform extract of *Colocasia gigantea* using solvent system chloroform: methanol in the ratio of 5:5, 6:4 and 7:3

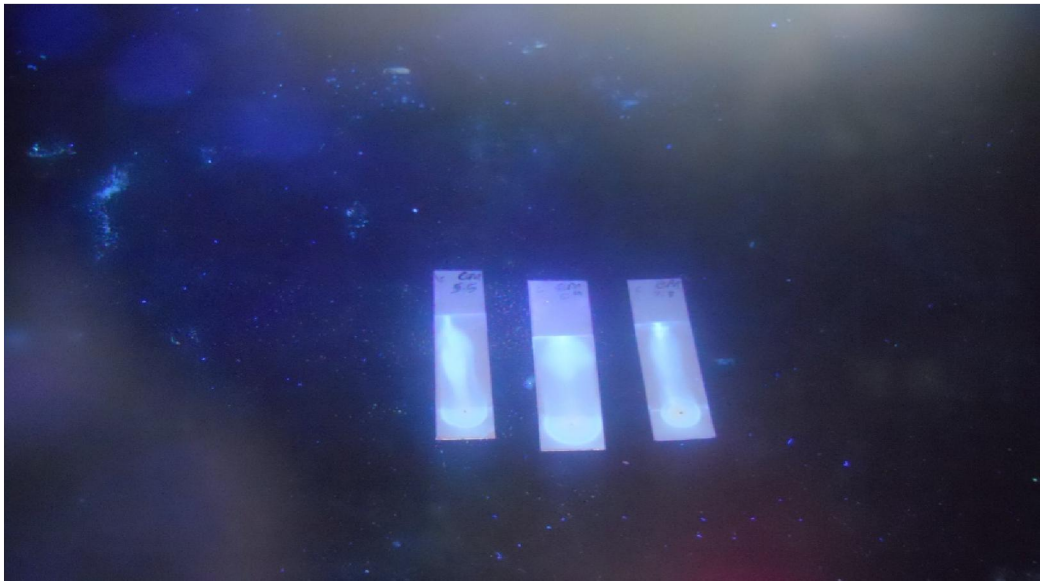

Figure 5: TLC Profile of chloroform extract of *Colocasia gigantea* under UV 365 nm using solvent system chloroform: methanol in the ratio of 5:5, 6:4 and 7:3

CHAPTER 3

FREE RADICAL SCAVENGING AND ANTIOXIDANT POTENTIAL OF DIFFERENT EXTRACTS OF *COLOCASIA GIGANTEA* (BLUME) HOOK. *f.*

CHAPTER 3

Abstract

Free radicals have been implicated in various diseases including cancer. The agents that can inactivate the formation of free radicals or scavenge free radicals may be of great potential to reduce the oxidative stress induced health disorders in humans. The leaves and rhizomes of Colocasia gigantea form part of human diet as they are consumed by humans as vegetable regularly. The present study was undertaken to study the free radical scavenging activity of Colocasia gigantea in vitro. The rhizomes of Colocasia gigantea were collected, shade dried, powdered and sequentially extracted in chloroform, ethanol, and water. The antioxidant activity of various extracts was evaluated by their ability to inhibit the generation of DPPH, hydroxyl ($^{\bullet}OH$), superoxide ($O_2^{\bullet-}$), $ABTS^{\bullet+}$ and nitric oxide ($NO^{\bullet}$) radicals in vitro. Total flavonoid and the total phenol contents were also determined to understand their role in free radical scavenging. The chloroform, ethanol, and aqueous extracts of Colocasia gigantea showed a concentration dependent inhibition in DPPH, $^{\bullet}OH$, $O_2^{\bullet-}$, $ABTS^{\bullet+}$ and $NO^{\bullet}$ radical generation. Different extracts of Colocasia gigantea showed the presence of polyphenols and flavonoids and inhibited the generation of DPPH, $^{\bullet}OH$, $O_2^{\bullet-}$, $ABTS^{\bullet+}$ and $NO^{\bullet}$ radicals. The C. gigantea scavenged DPPH, $^{\bullet}OH$, $O_2^{\bullet-}$, $ABTS^{\bullet+}$and $NO^{\bullet}$ radicals in a concentration dependent manner and this activity may be due the presence of various polyphenols and flavonoids in its rhizomes.

CHAPTER 3

1. INTRODUCTION

A free radical is an atom or molecule with an unpaired electron in its outer most orbit (Jesberger and Richardson, 1991; Halliwell and Gutteridge, 2006; Gutteridge and Halliwell, 2010), which is freely available for reaction. Such unpaired electrons make these species very unstable and highly reactive with other molecules (Karlsson, 1997) and they try to pair their electron(s) and generate a more stable compound. The oxygen derived radicals also known as Reactive Oxygen Species (ROS) are an important class of radicals that are produced in living system for various purposes (Miller *et al*., 1990). The ROS are dangerous species and are highly reactive with the molecules around them (Sharma and Clark, 1998; Sies 2017). ROS is a collective term, which includes not only the oxygen radicals (Ö and OH) but also some non-radical derivatives of oxygen, including hydrogen peroxide (H2O2), hypochlorous acid (HOCl) and ozone (O_3) (Sjodin *et al.*, 1990;Sies 2017).

The free radicals are usually produced in organisms that utilize oxygen for energy production, especially during oxidative phosphorylation in the mitochondria. Similarly, the reactive nitrogen species (RNS) are equally important in biological systems as they are involved in several cellular processes including cell signaling (Valko *et al.,* 2007; Kreuz and Fischle, 2016). Antioxidant means "against oxidation." An antioxidant is any substance that retards or prevents deterioration, damage or destruction by oxidation (Halliwell *et al.,* 1995). Antioxidants includes several organic substances, including vitamin C, E, and A (which is converted from beta-carotene), selenium and a group known as carotenoids (Dekkers *et al.,* 1996; Kaczmarski *et al.,* 1999).Despite the fact that organisms have in built safety

mechanisms to neutralized free radicals by different antioxidant molecules present in the cell, excess induction of ROS and RNS does occur. This excess generation of ROS and RNS may overwhelm the endogenous defence system and supplementation of exogenous of antioxidants may be essential to neutralize the additional induction of ROS and RNS since the increased induction of ROS and RNS leads to several inflammatory diseases, especially autoimmune disorders, rheumatoid arthritis, cataract, aging, cardiovascular, gastrointestinal and neurodegenerative diseases and cancer (Valko *et al.,* 2007; Bhattacharyya *et al.,* 2014; Kreuz and Fischle, 2016). The exogenous supply of antioxidants may be required to neutralize the deleterious effect of ROS/RNS and support the endogenous antioxidants system (Jagetia and Reddy, 2011). Currently available synthetic antioxidants like butylated hydroxyl anisole (BHA), butylatedhydroxy toluene (BHT), tertiary butylated hydroquinone and gallic acid esters have been suspected to cause negative health effects, which has led to strong restrictions on their use in humans. This indicates that there is a need of non-toxic naturally occurring antioxidants, which do not trigger adverse effects. Recently, there has been an upsurge of interest in the therapeutic potentials of medicinal plants as antioxidants in reducing free radical-induced tissue injury. Many plant extracts and phytochemicals have shown to possess free radical scavenging activities (Jagetia *et al.,* 2003) but generally there is still a need to find more information concerning the antioxidant potential of other plant species.

*Colocasia gigantea (*family: Araceae) also called giant elephant ear or Indian taro is 1.5-3 m tall herb with a whorl of large leaves at its apex. It bears a large, fibrous, and inedible corm. *C.gigantea* grows commonly in India, Thailand and other Southeast Asian countries (Manner, 2011). The leaf stalks are used as a vegetable in

some areas in, India, South East Asia and Japan. In India and Bangladesh, the tubers are cooked and used as a vegetable (Kay, 1987). In Thailand, *C. gigantea* is considered as a minor food crop and is mainly utilized as a stem vegetable. *C. gigantea*'s stalks are often used for making homemade Thai food called "Bon curry". In the Pacific islands, the tubers are cooked and eaten as a starch (Manner, 2011). In Thai traditional medicine, *C. gigantea* tuber is heated over a fire and is used to reduce "internal heat" (fever) and also for the treatment of drowsiness. The fresh or dried tubers mixed with honey are used in the treatment of phlegm in northern Thailand. Fresh tubers have been shown to ameliorate stomach problems, combat infection, and accelerate the healing of wounds. Recently the leaf and tuber extracts have been found to be cytotoxic to HeLa cells (Pornprasertpol *et al.,* 2015). The supplementation of antioxidants from dietary source will be of great importance than those given exogenously from other sources. Since not much information is available on *Colocasia gigantea* despite the fact that is commonly used as a vegetable in India and Southeast Asia and is part of human diet, the present study was undertaken to investigate the antioxidant potential of different extract of *Colocasia gigantea.*

2. MATERIALS AND METHODS

2.1. Chemicals

All the chemicals used were of analytical grade and Milli Q water was used for the entire analysis. 1,1-dimethylsulfoxide (DMSO), ascorbic acid, nitroblue tetrazolium (NBT), ethylenediamine tetraacetic acid (EDTA), trichloroacetic acid (TCA), sodium nitroprusside, and Griess reagent were procured from Sigma-Aldrich Chemical Co. Bangalore, India. Methanol, ethanol, sodium acetate, ferric chloride, Folin-Ciocalteau reagent, sodium carbonate, sodium hydroxide, sodium chloride,

potassium chloride, disodium hydrogen phosphate (anhydrous), potassium dihydrogen phosphate, aluminium chloride, potassium acetate, gallic acid, glacial acetic acid and acetyl acetone were procured from Merck India, Mumbai).

2.2. Preparation of extract

The non-infected and matured rhizomes of *Colocasia gigantea* were collected and cleaned and chopped into small pieces. The rhizome pieces were spread into the stainless steel trays for drying under shade at room temperature in dark in clean and hygienic conditions to avoid entry of insects, animals, fungus, and extraneous terrestrial materials. The exhaust and free air circulation was allowed. The dried rhizomes were powdered in an electrical grinder at room temperature. Usually 100 g of rhizome powder was extracted sequentially in chloroform, ethanol and water in a Soxhlet apparatus, evaporated to dryness under reduced pressure and stored at -80°C until use. The chloroform, ethanol and water extracts of *Colocasia gigantea* will be called as CGC, CGE and CGA henceforth.

3. ESTIMATION OF FREE RADICAL SCAVENGING *IN VITRO*

The scavenging activity of CGC, CGE and CGA was estimated using the following protocols:

3.1. DPPH free radical scavenging assay

The DPPH scavenging activity of CGC, CGE and CGA was carried out according to Leong and Shui (2002) with minor modifications. To 20, 40, 60, 80, 100, 120, 140, 160, and 180µg/ml CGC, CGE or CGA 1 ml of 0.1 mM DPPH in methanol was added. After thorough mixing, the mixture was kept in the dark for 30 min and the absorbance was measured at 523 nm using UV-VIS spectrophotometer (SW 3.5.1.0. Biospectrometer, Eppendorf India Ltd., Chennai).Methanol was utilized

for the baseline correction. The results have been compared with that of the control prepared as above without sample. Radical scavenging activity has been expressed as a percentage and calculated using the following formula:-

% Scavenging= ($A_{control}$-A_{sample})/ $A_{control}$X 100.

Where A_{sample} is the absorbance of the test sample and $A_{control}$ is the absorbance of the control.

3.2.Hydroxyl radical scavenging activity

Scavenging of the hydroxyl ($^{\bullet}OH$) free radical was determined by the earlier described method (Halliwell, 1987). Briefly, the reaction mixture contained deoxyribose (2.8 mM), KH_2PO_4-NaOH buffer, pH 7.4 (0.05 M), $FeCl_3$ (0.1 mM), EDTA (0.1 mM), H_2O_2 (1 mM), ascorbate (0.1 mM) and 20, 40, 60, 80, 100, 120, 140, 160, 180 or 200 µg/ml CGC, CGE or CGA in a final volume of 2 ml. The reaction mixture was incubated for 30 min at ambient temperature followed by the addition of 2 ml trichloroacetic acid (2.8% w/v) and thiobarbituric acid. The reaction mixture was kept in a boiling water bath for 30 min, cooled and the absorbance was read at 532 nm in a UV-VIS spectrophotometer. The results have been expressed as gallic acid equivalent which was used as a standard.

3.3. Superoxide anion scavenging activity

Superoxide free radicals formed by alkaline DMSO react with NBT to produce coloured diformazan. Scavenging of the superoxide ($O_2^{\bullet-}$) anion radical was measured using a modified method (Hyland *et al.,* 1983). Briefly, the reaction mixture contained 0.2 ml NBT (1 mg/ml in DMSO) and 0.6 ml of various concentrations (20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 µg/ml) of CGC, CGE or CGA 2 ml of alkaline DMSO (1 ml DMSO containing, 5 mM NaOH in 0.1 ml

water). The blank consisted of pure DMSO instead of alkaline DMSO. The absorbance was recorded at 560 nm in a UV/VIS spectrophotometer. The antioxidant capacity of the CGE based on its ability to inhibit formazan formation, which has been expressed as mg ascorbic acid equivalent /100 g of extract.

3.4. ABTS scavenging activity

ABTS scavenging activity of CGC, CGE or CGA was carried out as described earlier (Re *et al.,* 1999). Briefly, 37.5 mg of potassium persulphate was dissolved in 1 ml of distilled water. 44 µl of this solution was added to 9.7 mg of ABTS dissolved in 2.5 ml of distilled water to prepare ABTS solution. The ABTS solution was allowed to stand in the dark at room temperature for 12-16 hours. The working solution consisted 1 ml of ABTS solution, 88 ml of 50% ethanol. 25 µl of different concentrations (20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 µg/ml) of the different extracts of *C. gigantea* were mixed with 250 µl of the working ABTS solution and allowed to react for 4 minutes. The absorbance was then measured at 734 nm in a UV-VIS spectrophotometer. Gallic acid was used as the standard antioxidant and the activity was expressed as gallic acid equivalent. The percentage scavenging activity was calculated as follows:

Scavenging (%) = Control O.D – Sample O.D/ Control O.D x 100.

3.5. Nitric oxide scavenging activity

The nitric oxide scavenging activity was estimated by spectrophotometric method (Marcocci *et al.,* 1994). Briefly, sodium nitroprusside (5 mM) in phosphate buffer saline was mixed with 20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 µg/ml CGC, CGE or CGA and incubated at 25°C for 150 min. The samples were mixed with Greiss reagent (1% sulfanilamide, 2% H_3PO_4, and 0.1% napthylenediamine

dihydrochloride). The absorbance of the chromophore formed during diazotization of nitrite with sulfanilamide and subsequent coupling with napthylenediamine was read at 546 nm using a UV-VIS spectrophotometer. The inhibition of nitric oxide formation was determined with respect to standard potassium nitrite treated in the same way with Greiss reagent. The results have been expressed as potassium nitrite equivalent which has been used as a standard.

3.6. Determination of Total phenolic contents

The total phenolic contents were estimated by Folin-Ciocalteau reagent (McDonald *et al.,* 2001), where 20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 µg/ml CGC, CGE or CGA after dilution or gallic acid (standard phenolic compound) were mixed with Folin-Ciocalteau reagent (5 ml, 1:10 diluted with distilled water) and aqueous Na2CO3 (4 ml, 1M). The mixture was allowed to stand for 15 minutes and the total phenolic contents were measured at 756 nm with a UV-VIS spectrophotometer. The total phenol contents are expressed in terms of gallic acid equivalent (mg/100 g of extracts).

3.7. Total Flavonoids Determination

The total flavonoid contents in CGC, CGE or CGA were estimated using Aluminum chloride method as described earlier (Chang *et al.,* 2002). Different concentrations of *C. gigantea* extract (20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 µg/ml) were separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1M potassium acetate and 2.8 ml distilled water and incubated at room temperature for 30 min. The absorbance was recorded at 415 nm with a UV-VIS spectrophotometer. Quercetin was used as the standard and the results have been expressed as quercetin equivalent.

CHAPTER 3

4. RESULTS:

4.1. DPPH radical scavenging activity

Various extracts of *Colocasia gigantea* showed a concentration dependent rise in the scavenging of DPPH radicals as indicated by the discolouration of DPPH which is purple in colour. Maximum scavenging was observed at a concentration of 140 µg/ml for CGC, CGE or CGA that ranged between 50.41±0.30, 51.08±0.68 to 51.2±0.23 mg of ascorbic acid equivalent, respectively (Table 1and Figure 1).

4.2. Hydroxyl Radical Scavenging activity

Different extracts of *C. gigantea* inhibited the generation of hydroxyl radical in a concentration dependent manner and a maximum inhibition in OH generation was observed at 200µg/ml for chloroform, ethanol and aqueous extracts, respectively (Figure 2). When the efficiency of scavenging was determined with respect to gallic acid equivalent the most effective extract was CGA followed by, CGE and CGC, respectively (Figure 2).

4.3. Superoxide anion scavenging activity

The chloroform, ethanol and aqueous extracts of *C. gigantea* showed a concentration dependent rise in the inhibition of superoxide radical generation up to a concentration of 140µg/ml that declined thereafter (Figure 3). The maximum effect was observed for ethanol extract which scavenged superoxide radical more efficiently than the other two extracts and this was 59.17±0.23 mg ascorbic acid equivalent, whereas it was 51.02±0.07mg and 50.63±0.11 mg ascorbic acid equivalent for chloroform and aqueous extracts, respectively (Table 3 and Figure 3).

4.4. ABTS scavenging activity

The different extracts of *C. gigantea* showed a concentration dependent rise in the scavenging activity of the ABTS free radicals up to a concentration of 140µg/ml CGE and CGC and declined thereafter, whereas a maximum scavenging effect for aqueous extract was recorded at 180 µg/ml and a decline thereafter (Figure 4).

4.5. Nitric oxide scavenging activity

Various extracts of *C. gigantea* showed a concentration dependent increase in the scavenging activity of nitric oxide radicals and a highest scavenging of $NO^{\bullet}$ was observed for 140µg/ml for chloroform and ethanol, whereas and for aqueous extracts showed the highest scavenging activity at 120µg/ml (Figure 5). Among all the three extract the best effect was observed for CGA that revealed maximum activity at a lower concentration than the other two extract (Figure 5).

4.6 Total phenolic contents

The presence of phenolic compounds in the CGC, CGE and CGA was estimated as total phenol contents that increased in a concentration dependent manner up to 200µg/ml for chloroform, ethanol and aqueous extracts, respectively (Figure 6). The CGA showed the least amount of total phenols when compared to CGE and CGC, where the amount was maximum (Figure 6).

4.7. Total flavonoid contents

The total flavonoid contents in CGC, CGE and CGA increased in a concentration dependent manner up to 200µg/ml equivalent to gallic acid contents (Figure 7). The maximum amount of total flavonoid was estimated for CGC followed by CGA, whereas it was least for CGE (Figure 7).

CHAPTER 3

DISCUSSION:

The oxidative stress is the price organisms have to pay for using oxygen as a chemical energy source that is required for various activities. The oxidative stress is induced due to the production of free radicals during various metabolic activities and respiration in particular. The cells are equipped with repertoire of antioxidant or antioxidant enzymes that usually take care of the normal oxidative stress induced during respiration however in situation of excess oxidative stress it may not be possible for the endogenous antioxidant system to passivate it. Moreover, generation of excess oxidative stress has been indicated as a causative factor of several disorders including, aging, autoimmune, benign oral, cardiovascular, kidney, liver, intestine, and Alzheimer diseases and arthritis, diabetes and cancer (Halliwell, 1994; 2012; Pham-Huy *et al.,* 2008; Kesarwala *et al.,* 2016). It is also known that external supplementation with antioxidants have been helpful in reducing the risk of oxidative stress. It will be better if the antioxidants come from the dietary sources. The *Colocasia gigantea* or Indian taro is part of diet and it is consumed frequently during the season. Therefore, the present study was undertaken to investigate the antioxidant activity of various extracts of *Colocasia gigantea* in vitro.

DPPH (1, 1-diphenyl-2-picrylhydrazyl) assay is one of the simple and convenient technique to estimate the antioxidant property of any agent. DPPH is a stable free radical by virtue of the delocalization of the free electron over the molecule, which is violet coloured, and faints into the yellow coloured congener DPPH-H once it accepts an electron donated by the antioxidant and subsequently get reduced with a high λ-shift in the visible spectra (from 520 nm to 330 nm). This

redox process was first reported by Goldschmidt and Renn (1922). All extracts of *Colocasia gigantea* scavenged DPPH free radical in a concentration dependent manner up to 140 µg/ml. Somewhat similar results have been reported for *Agele marmelos*, *Croton caudatus*, *Milletia pachycarpa*, *Schima wallichi, Eleagnus caudata, Castanopsis indica* and *Dysoxylum gobara, oroxylum indicium* (Jagetia *et al.,* 2003; Lalrinzuali *et al.,* 2014, 2015) The other phytochemicals naringin, and mangiferin have been reported to scavenge DPPH free radicals in a concentration dependent manner (Jagetia and Baliga, 2003; Jagetia and Venkatesha, 2005).

The hydroxyl free radical is highly reactive species, which reacts in the close vicinity of its formation (Pastor *et al.,* 2000). During like respiration superoxide radical is converted into H_2O_2 which is a highly toxic and oxidizing agent. Despite H_2O_2 is not very reactive, it becomes highly toxic to the cell due to its ability to generate hydroxyl radical in the cells in presence of metals by Haber Weiss and/or Fenton reaction (Halliwell, 2006; Valko *et al.,* 2007; Yang *et al.,* 2014). The hydroxyl radical is capable of inducing detrimental effects on the important macromolecules including proteins and nucleic acids. It reacts with DNA leading to base and sugar damages (Tsunoda, 2010; Verlackt *et al.,* 2015). Hence, neutralization of hydroxyl radical is crucial to protect cells from its deleterious effects. The various extracts of *Colocasia gigantea* inhibited the generation of OH radicals in a concentration dependent manner and hence it may be a useful agent to inactivate this radical in vivo. Many other plant extracts and flavonoids have been found to scavenge hydroxyl free radicals in a concentration dependent manner (Jagetia *et al.,* 2003 a,b; 2012; Jagetia and Venketasha, 2005; Shantabi *et al.,* 2014, Lalrinzuali *et al.,* 2015).

CHAPTER 3

The superoxide free radical is an intermediate during cellular respiration which is produced as a result of incomplete metabolicm of oxygen (Kirkinezosa and Morae, 2001). It has been reported to paly important role in cell signaling (Weidinger and Kozlov, 2015). However, the superoxide anion produces H_2O_2, which in turn generates hydroxyl free radicals in the presence of metals leading to pathologic alteration of several important macromolecules in the cell (Turrens, 2003). Therefore, neutralization of superoxide radical will be able to arrest the chain of ROS generation and protect the cells from the oxidative stress. The various extracts of *C. gigantea* have been found to inhibit the production of superoxide radical in a concentration dependent manner. Other plant extracts and some flavonoids have been reported to scavenge the superoxide anion free radical earlier (Jagetia *et al.,* 2003a; 2003b; Jagetia and Venkatesha, 2005; Jagetia *et al.,* 2012; Shantabi *et al.,* 2014, Lalrinzuali *et al.,* 2015).

The nitric oxide radical (NO•) is a labile molecule and it is generated in mammalian cells as a byproduct of respiration. It is also used by neutrophils to eliminate invading bacteria (Valko *et al.,* 2007). NO• also plays an important role in signal transduction and nerve conduction. However, excess production of NO• is toxic, especially after reaction with oxygen or superoxide anion radicals and the reaction products includes NOx and ONOO- (peroxynitrite). These products are able to inflict severe cellular damage (Beckman *et al.,* 1990; Radi *et al.,* 1991; Lipton *et al.,* 1993: Roberts *et al.,* 2009). The various extracts of *C. gigantea* reduced the generation of NO• in a concentration dependent manner. Several plant extracts and plant formulations have also been reported to scavenge NO• in a concentration dependent manner (Jagetia *et al.,* 2003b; 2012). Similarly, some of the plant flavonoids including naringin and

mangiferin have been reported to scavenge nitric oxide free radical in a concentration dependent manner earlier (Jagetia *et al.,* 2003a; 2012; Jagetia and Venkatesha, 2005).

The $ABTS^{\bullet+}$ chromophore was produced through the reaction between ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) and potassium persulfate which is indicated by blue/green colouration. The addition of antioxidants to the pre-formed radical cation reduces the ABTS, indicated by the discoloration of the blue/green colour. The extent of decolorization was expressed as the percentage inhibition of the $ABTS^{\bullet+}$ (Re *et al.,* 1999). This trapping of ABTS derived radical cation ($ABTS^{\bullet+}$) by free radical scavengers is a commonly employed method to evaluate the total charge of antioxidants present in complex mixtures (Aliaga and Lissi, 1998). The inhibitory action of $ABTS^{\bullet+}$indicates the antioxidant potential of any chemical agent. The different extracts of *C. gigantea* did inhbit the generation of $ABTS^{\bullet+}$ radical in a dose dependent manner. A similar effect has been observed with *Agele marmelos, Syzygium cumini* earlier (Jagetia *et al.,* 2003; Jagetia and Shetty 2012).

The exact mechanism of radical scavenging of free radicals by *C. gigantea* is not known. However, the free radical scavenging and antioxidant activity of *C .gigantea* may be due to the presence of various phytochemicals like polyphenols and flavonoids, which may be able to donate or accept electron thus neutralizing their oxidative effects. Plants produce phenolic compounds and flavonoids in particular as secondary metabolites that help plants in pollination, to ward off against fungal attacks and also give attractive colours to flowers (Middleton and Chithan, 1993; Harborne and Baxter, 1999; Harborne and Williams, 2000). These flavonoids have

been reported to exert a conducive effect on human health owing to their free radical scavenging ability and antioxidant nature.

CONCLUSIONS:

The present study showed that all the extracts of *C.gigantea* showed a concentration dependent inhibition of free radicals. These activities of *C. gigantea* may be due to the presence of various phenolic compounds and flavonoids. Therefore, *C. gigantea* could be a potential source of natural antioxidant which may act as therapeutic agent in preventing or slowing down the progression of oxidative stress related degenerative diseases.

CHAPTER 3

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Table 1: Percentage scavenging activities of DPPH and Hydroxyl radical of different extracts of *Colocasia gigantea*. Values were expressed as Mean± SEM, N=5.

Conc. (µg/ml)	DPPH			HYDROXYL RADICAL		
	H_2O	EtOH	$CHCl_3$	H_2O	EtOH	$CHCl_3$
20	26.75±0.38	36±0.14	32.33±0.24	22.16±0.15	22.87±0.13	24.05±0.15
40	30.83±1.04	41±0.14	34.6± 0.11	23.76±0.18	23.75±0.13	26.33±0.10
60	34.25±0.38	44±0.38	37.13±0.17	26.51±0.28	30.22±0.15	30.22±0.26
80	39.08±0.22	48.5±0.14	39.46±0.24	32.47±0.37	33.90±0.13	34.44±0.18
100	41.25±0.62	48.91±0.60	41.33±0.40	40.32±1.28	36.77±0.07	35.42±0.18
120	43.91±0.36	54.08±0.68	46.6±0.23	38.31±0.23	41.31±0.10	36.89±0.23
140	50.41±0.30	51.33±0.65	51.2±0.23	48.38±0.39	44.75±0.18	39.07±0.10
160	45.91±0.44	49.91±0.22	46.86±0.35	49.86±0.56	46.66±0.10	42.87±0.43
180	44.41±0.22	47.83±0.50	42.8±0.11	57.83±0.24	51.07±0.28	46.25±0.91
200	42.91±0.20	45.91±0.22	40.2±0.07	60.94±0.36	56.39±0.13	51.91±0.10
220	-	-	-	54.31±0.54	56.39±0.13	46.97±0.10
240	-	-	-	48.32±0.34	48.50±0.18	42.30±0.53

Table 2: Percentage scavenging activities of Superoxide and ABTS radicals of different extracts of *Colocasia gigantea*. Values were expressed as Mean± SEM, N=5.

Conc. (µg/ml)	SUPEROXIDE RADICAL			ABTS		
	H_2O	EtOH	$CHCl_3$	H_2O	EtOH	$CHCl_3$
20	26.06±0.11	11.16±0.45	25.61±0.13	23.87±0.19	26.12±0.33	24.35±0.62
40	28.86±0.08	14.29±0.34	28.13±0.07	25.35±0.07	32.96±0.12	26.79±0.11
60	29.22±3.24	26.20±0.60	31.26±0.04	28.11±0.05	35.43±0.09	30.70±0.08
80	37.89±0.37	31.46±0.64	33.89±0.04	30.02±0.15	37.85±0.29	38.34±0.05
100	43.76±0.31	38.88±0.87	37.31±0.04	33.42±0.30	41.61±0.22	38.50±0.22
120	49.45±0.30	52.15±0.19	47.86±0.10	36.90±0.05	43.18±0.19	45.70±0.17
140	50.63±0.11	59.17±0.23	51.02±0.07	42.73±0.45	46.88±0.04	48.17±0.13
160	47.53±0.11	56.87±0.19	48.92±0.09	49.34±0.10	50.28±0.52	51.47±0.14
180	45.87±0.08	54.27±0.16	47.83±0.16	53.75±0.07	47.39±0.09	45.98±3.25
200	43.85±0.11	52.33±0.20	46±0.05	49.37±0.22	44.64±0.07	42.15±2.90

Table 3: Percentage scavenging activities of Nitric oxide of different extracts of *Colocasia gigantea*. Values were expressed as Mean± SEM, N=5.

Conc. (µg/ml)	NITRIC OXIDE RADICAL		
	H_2O	EtOH	$CHCl_3$
20	19.59±0.77	22.09±0.67	25.19±0.45
40	22.80±0.50	26.74±0.67	27.82±0.69
60	34.50±0.77	28.68±1.02	31.49±0.45
80	42.10±0.50	32.94±1.02	38.58±0.45
100	46.49±0.50	39.92±1.02	40.94±0.45
120	54.97±0.77	45.34±0.67	48.03±0.45
140	50.87±0.50	53.10±1.02	54.85±0.69
160	49.12±0.50	47.28±1.02	47.50±0.94
180	42.69±0.77	33.72±0.67	43.56±0.69
200	35.08±1.01	28.68±1.02	30.97±0.69
220	32.49±0.77	26.74±0.67	27.56±0.45
240	30.69±0.50	23.68±1.02	24.85±0.69

Table 4: Percentage scavenging activities of Total Phenols and Total Flavanoids of different extracts of *Colocasia gigantea.* Values were expressed as Mean± SEM, N=5.

Conc. (µg/ml)	TOTAL PHENOLS			TOTAL FLAVANOIDS		
	H_2O	EtOH	$CHCl_3$	H_2O	EtOH	$CHCl_3$
20	84.16±2.20	102.5±1.44	114±1.73	271.15±8.96	189.06±20.36	280.60±15.79
40	90±1.44	162.5±12.82	184±2.64	353.83±19.74	213.93±4.97	331.35±5.17
60	113.33±7.12	204.16±2.20	258±1.73	385.58±15.13	231.35±4.30	373.15±10.76
80	147.5±1.44	240±1.44	264±1.73	447.78±4.30	253.74±4.30	420.91±10.34
100	162.5±1.44	289.16±2.20	310±3.60	502.50±8.96	308.47±6.58	459.72±7.89
120	161.66±4.63	333.33±2.20	365±2.64	537.33±4.30	350.76±8.61	501.51±5.17
140	182.5±1.44	355±1.44	424±2.64	567.18±4.30	393.05±8.96	588.08±16.62
160	205±1.44	387.5±1.44	488±2.64	634.35±12.92	432.85±4.30	680.62±5.17
180	222.5±1.44	430.83±3.63	527±2.64	684.10±6.58	457.73±6.58	761.22±15.51
200	265±1.44	505.83±2.20	576±1.73	726.39±6.5	8497.53±6.58	829.88±7.89

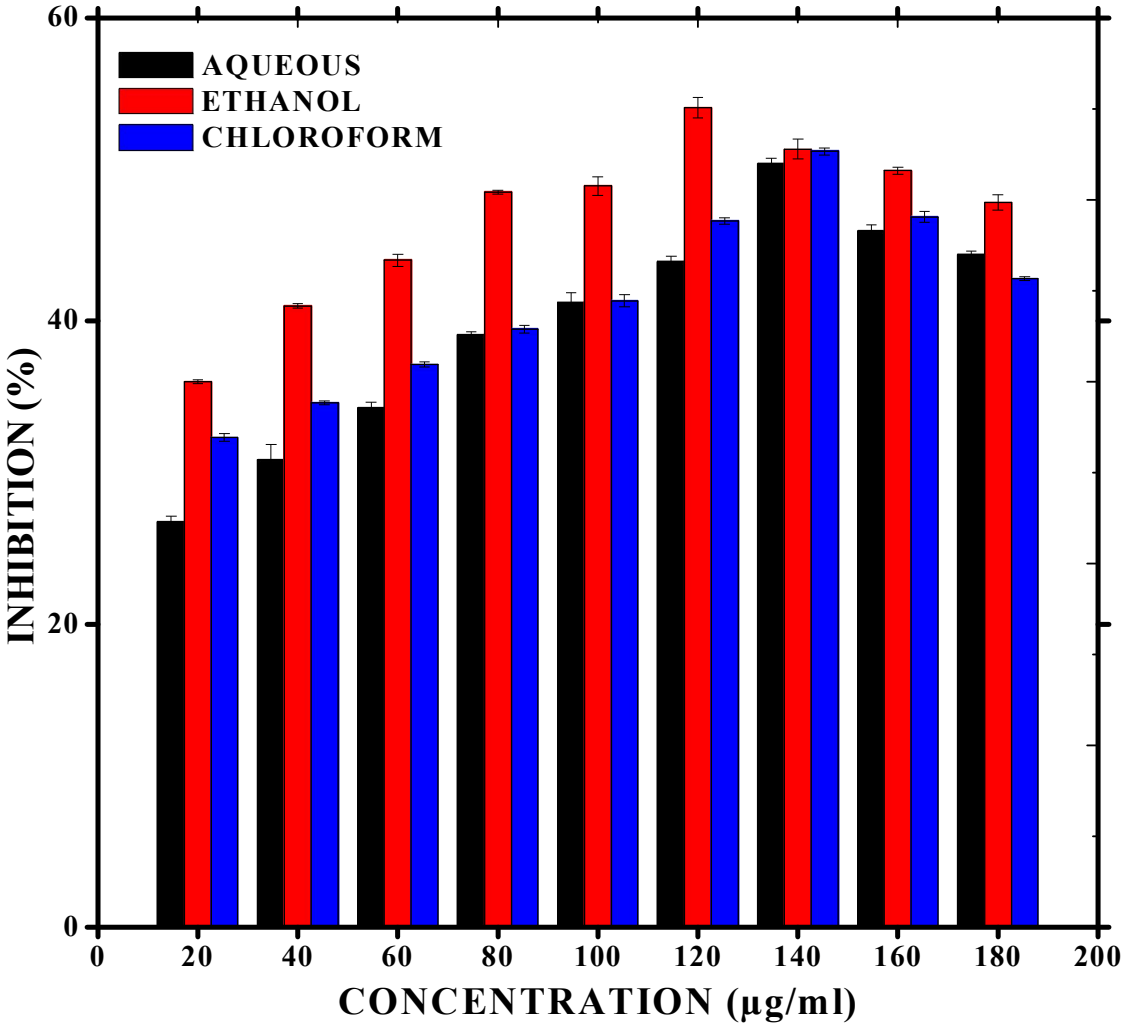


Figure 1: Different extracts of *Colocasia gigantean* on DPPH radicals scavenging activity (20-200µg/ml). Values were expressed as Mean± SEM, N=5.

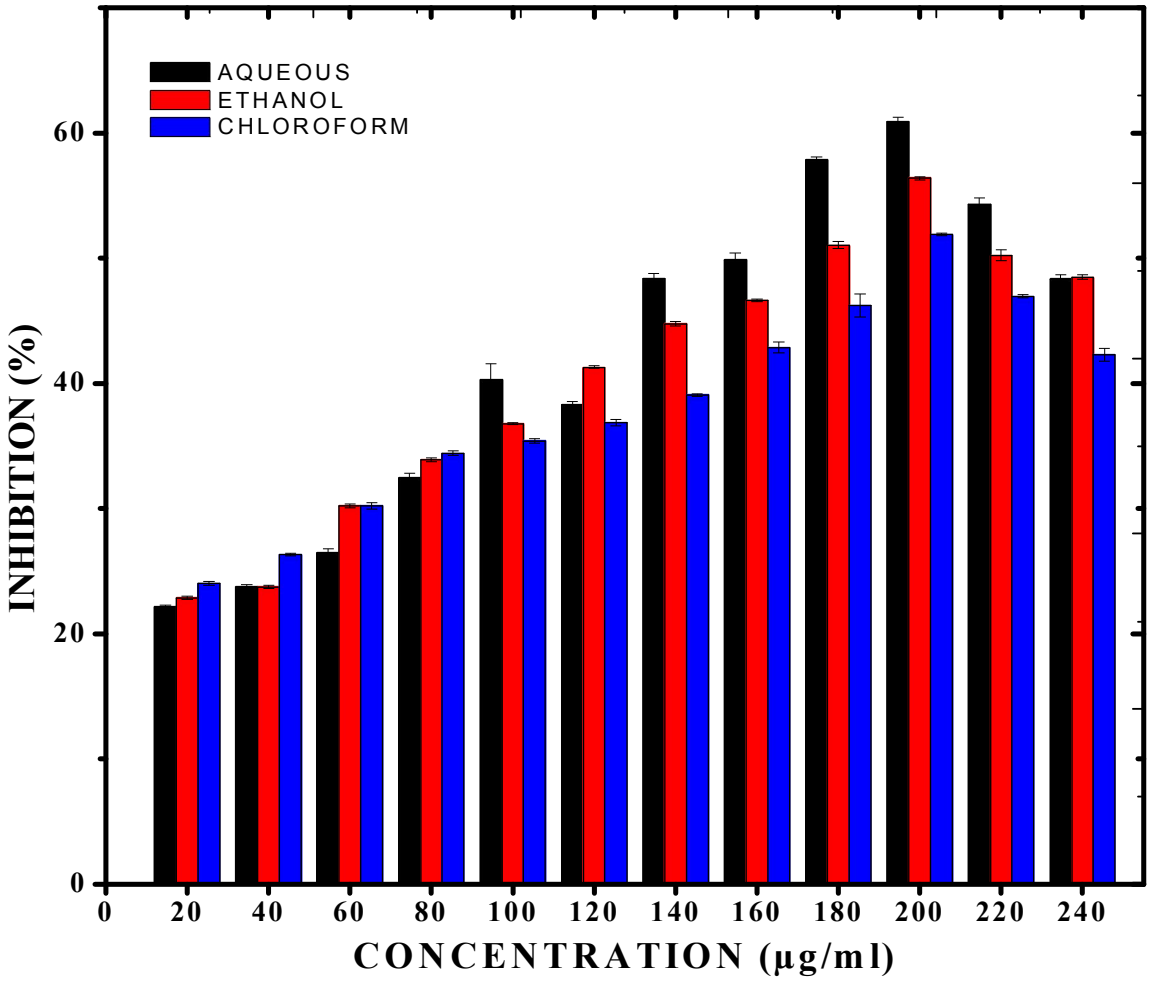


Figure 2: Hydroxyl radicals scavenging activity of different extracts of *Colocasia gigantea* expressed as Gallic acid equivalent (20-240µg/ml). Values were expressed as Mean± SEM, N=5.

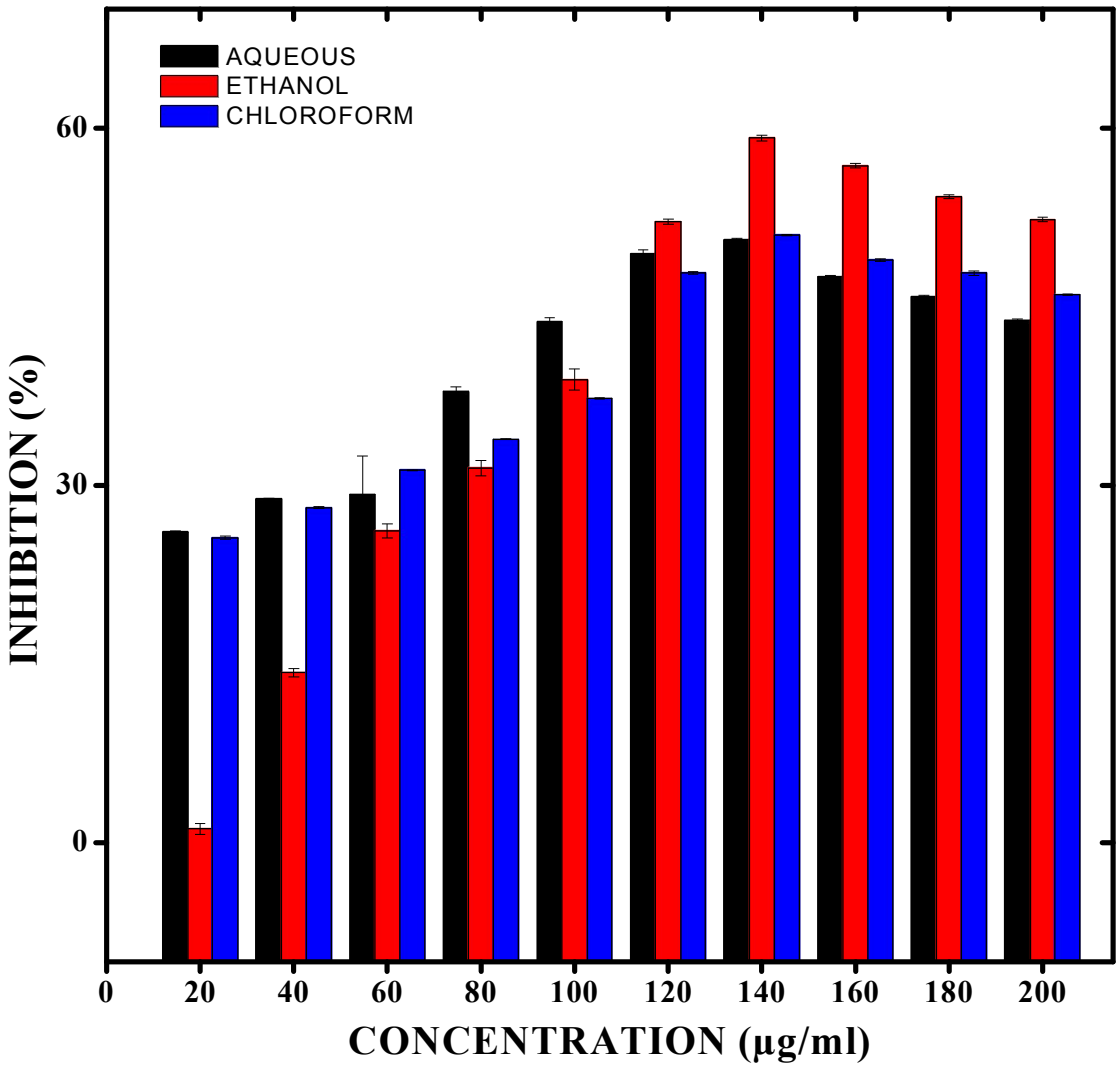


Figure 3: Superoxide radicals scavenging activity of different extracts of *Colocasia gigantea* (20-200µg/ml). Values were expressed as Mean± SEM, N=5.

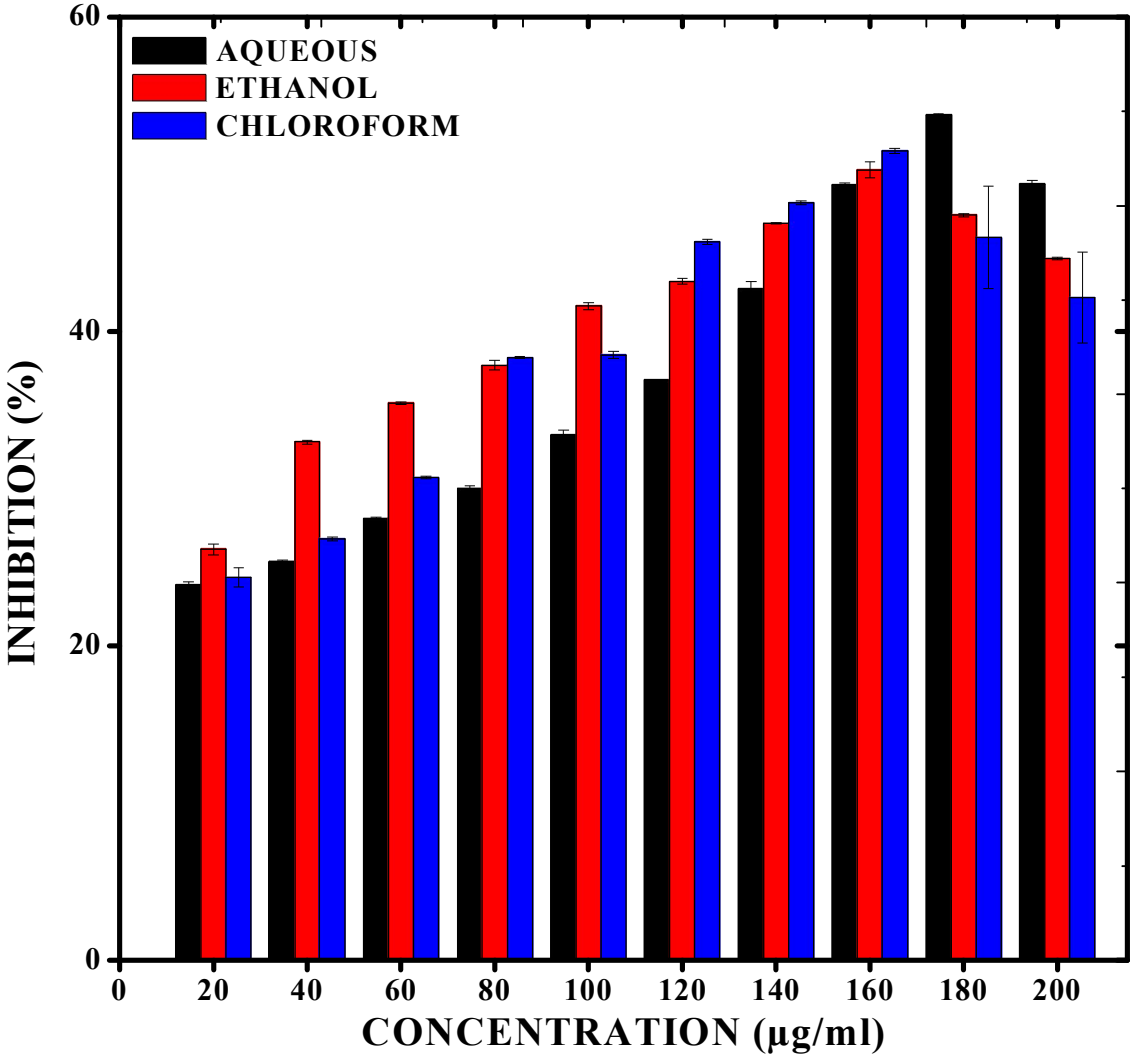


Figure 4: ABTS radicals scavenging activity of different extracts of *Colocasia gigantea* (20-200µg/ml). Values were expressed as Mean± SEM, N=5.

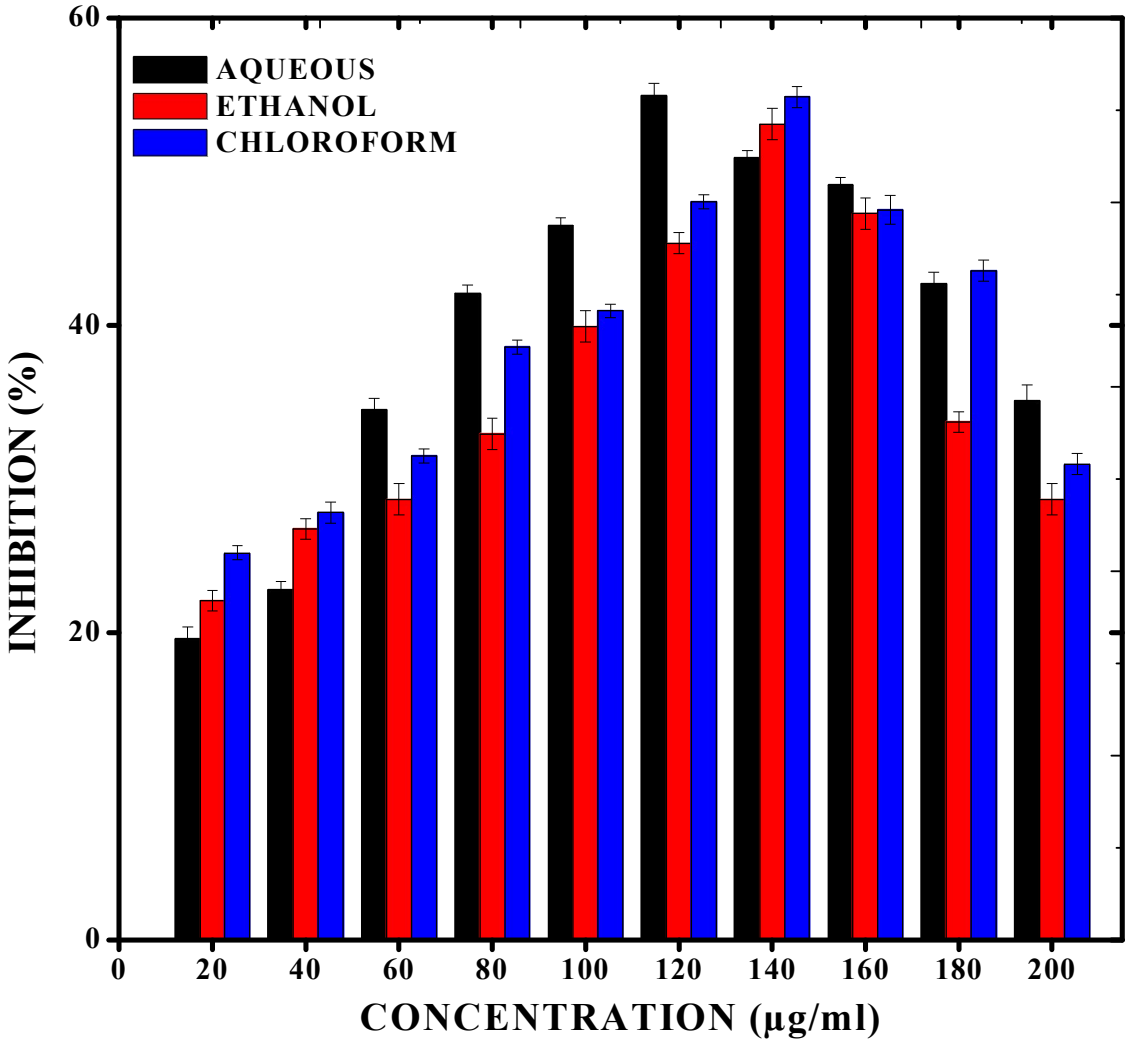


Figure 5: NO radicals scavenging activity of different extract of *Colocasia gigantea* (20-200µg/ml). Values were expressed as Mean± SEM, N=5.

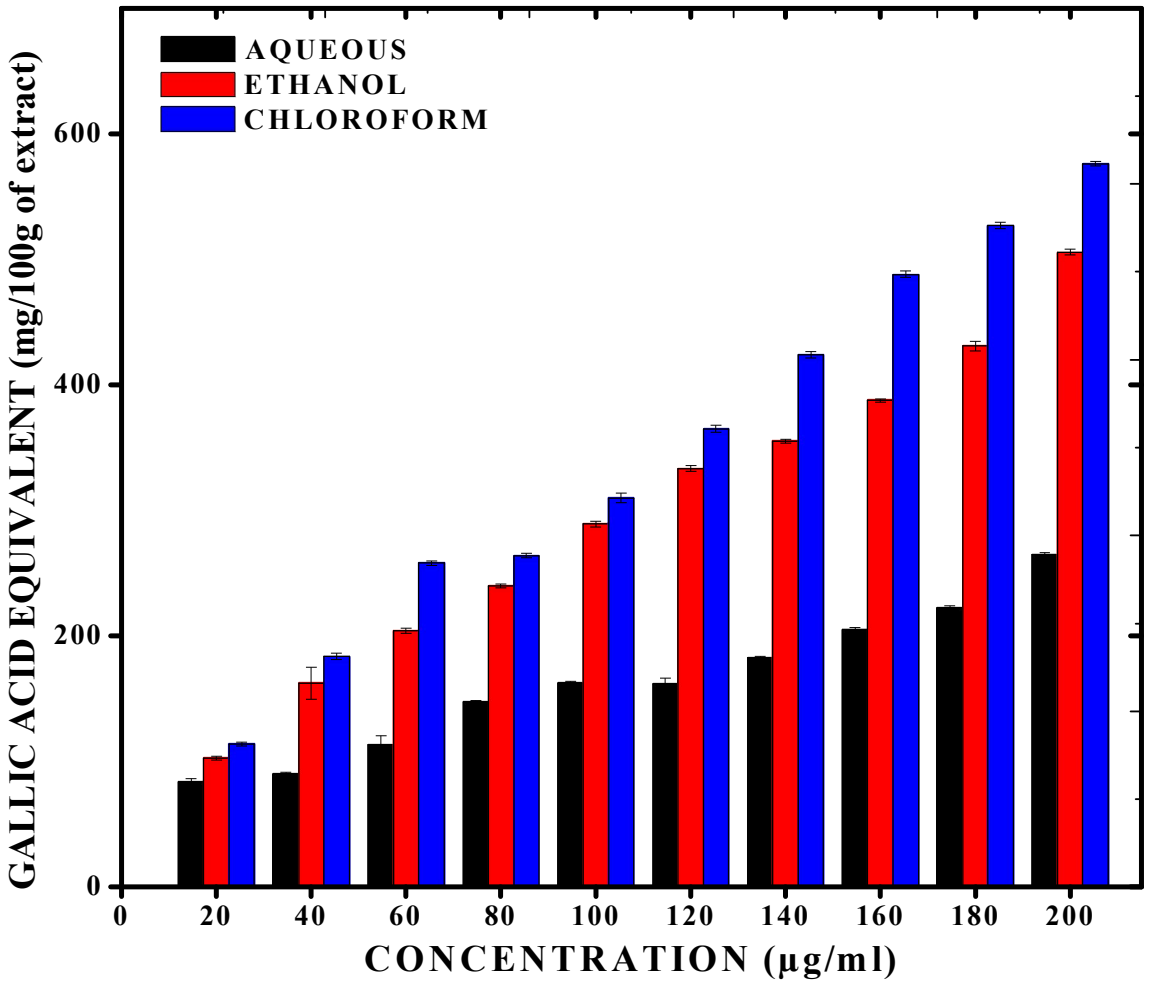


Figure 6: Total phenols contents of different extracts of *Colocasia gigantea* (20-200µg/ml). Values were expressed as Mean± SEM, N=5.

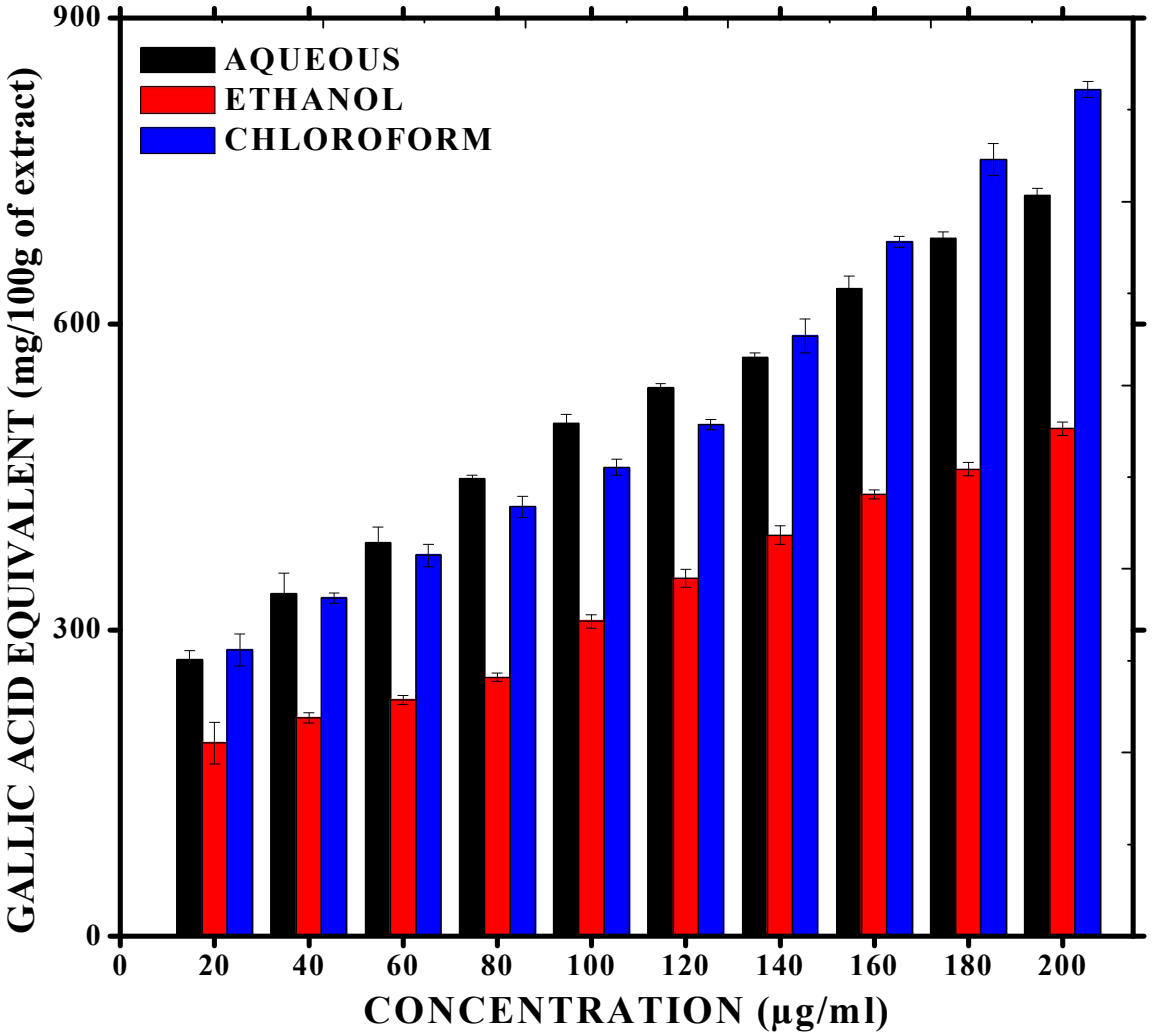
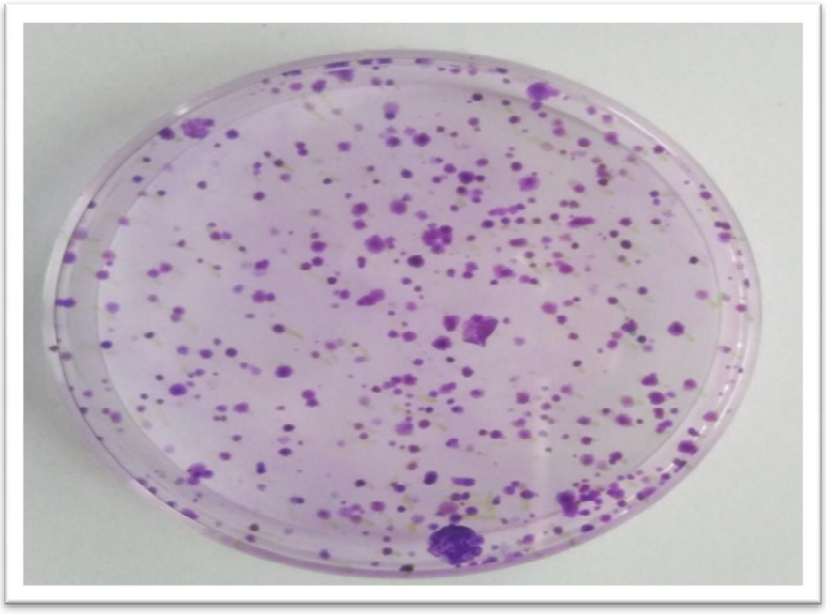


Figure 7: Total flavonoids contents of different extracts of *Colocasia gigantea* (20-200µg/ml). Values were expressed as Mean± SEM, N=5.

CHAPTER 4



ANTICANCER ACTIVITY OF *COLOCASIA GIGANTEA* (BLUME) HOOK. *f.* IN CULTURED CELL LINES

Chapter 4

Abstract

Evaluation of cytotoxic effects of the ethanol extract of Colocasia gigantea (CGE) in HeLa and V79 cells by MTT assay showed a concentrations dependent rise in the cytotoxicity. The maximum cytotoxicity was observed at 300 and 200 µg/ml ethanol extract of Colocasia gigantea for HeLa and V79 cells, respectively. Assessment of treatment duration revealed that cytotoxic effect of ethanol extract of Colocasia gigantea was marginal increased with treatment duration. Treatment of HeLa cells with different concentrations of ethanol extract of Colocasia gigantea reduced the clonogenicity of cells in a concentration dependent manner, which reached a nadir at 300µg/ml. To understand the biochemical mechanism of action, the HeLa cells were treated with different concentrations of ethanol extract of Colocasia gigantea and contents of glutathione and activities of the glutathione-s-transferase and catalase and lipid peroxidation were determined. The ethanol extract of Colocasia gigantea reduced the glutathione concentration and activities of the glutathione-s-transferase and catalase in a concentration and time dependent manner and greatest reduction was observed at 6 h post treatment, whereas lipid peroxidation increased in a concentration and time dependent manner. The ethanol extract of Colocasia gigantea induced cytotoxicity and reduced the reproductive integrity of HeLa cells. The cytotoxicity of ethanol extract of Colocasiagiganteamay be due to elevated lipid peroxidation and reduced concentration of glutathione and glutathione-s-transferase and catalase activities.

Chapter 4

1. INTRODUCTION

Despite availability of state or art treatment regimen cancer still remains one of the leading causes of death in both the developed as well as in the under developed countries (Torre *et al.,* 2012). It is estimated that one in every eight deaths is due to cancer (Khazir *et al.,* 2014). Cancer is a multifaceted disease and with improving health facilities and increased life span more cancer cases are coming to light than ever before. This has also increased the mortality rates and cancer deaths are of major concern globally (Siegel *et al.* 2017). Apart from many synthetic drugs the natural products have also immensely contributed to the paraphernalia of chemotherapeutic drugs. The fact is that 80% of the global population still dependend on plants and natural product for their healthcare proves the importance of plants as a major source of medicine. The one third of the all drugs approved by Federal Drug Adminstration, USA has been natural products (Patridge *et al.,* 2016), This reemphasizes the importance of plants and natural products in healthcare and new drug discovery. The evaluation of natural products could provide a new breakthrough in cancer treatment and new technologies are being explored for obtaining novel compounds from biodiversity of nature. The pharmaceutical industry has a continuing need to find new and better chemical compounds to develop as drugs for human healthcare (Newman and Cragg, 2014; 2016). Many drugs used for the treatment of different diseases including cancer are obtained from natural products (Mathieu *et al.,* 2015) and plants provide a major platform for design and new drug discovery. About 75 % of the registered small anticancer molecules since the 1940s have drug discover form the complex secondary metabolites synthesized

by plants. Therefore it is necessary to screen diverse plants for their anticancer activity in the hope that there will be a time one it may be possible to come across some biomolecules that will treat cancer effectively with lesser adverse side effects. *Colocasia gigantea* (family Araceae), also known as Giant Elephant ear or Indian taro, is a stemless plant producing large leaves with underground rhizomes. The rhizomes and the stalks are eaten as a vegetable and the leaves are eaten raw with pomegranate in India. In Thailand, *C. gigantea* tubers are heated over fire and consumed as a medicine (Manner, 2011). It is used to treat drowsiness and to reduce internal heat. The tuber reduces stomach problems, cure infection and heals wounds. Fresh or dries tubers are being used in the treatment of phlegm along with honey (Essence of the Agriculture, Songkla University; 2006). It is also used in the treatment of tuberculosis and constipation in Hawaii (Kokua1977). Juice of taro are said to arrest arterial haemorrhage (Drury, 1873). The information regarding the anticancer activity of *Colocasia gigantea* is lacking and it is used as a diet, which indicates that if is found to kill cancerous cells it may be a useful paradigm to fight cancer. Therefore, the present study was undertaken to study the antitumour activity of *Colocasia gigantea in vitro.*

2. MATERIALS AND METHODS

2.1. Chemicals

Doxorubicin was supplied by Getwell Pharmaceuticals, Gurgaon, India. Reduced glutathione (GSH), 1-chloro-2,4-dinitronbezene (CDNB), 5,5'dithio 2-nitrobenzoic acid (DTNB), ethylenediamine tetra-acetic acid (EDTA), bovine serum albumin (BSA), thiobarbituric acid (TBA), crystal violet were obtained from Sigma

Chemical Co. (Bangalore, India). Trichloroacetic acid (TCA), hydrogen peroxide (H_2O_2)were procured from SD Fine Chemicals, Mumbai, India, whereas disodium hydrogenphosphate (Na_2HPO_4), Tris buffer (Tris (hydroxymethyl) aminomethane and ammonium oxalate were purchased from Merck India Limited, Mumbai, India. Trypsin EDTA 1X, MTT (3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide), MEM medium, fetal bovine serum (FBS), and reduced nicotinamide adenine dinucleotide (NADH) were requisitioned from HiMedia, Mumbai, India.

2.2. Collection of rhizomes and extraction

The matured and non-infected rhizomes of *Colocasia gigantea* (family-Araceae) were collected from Manipur. The plant was identified by PG Department of Botany, DM College, Imphal-West, Manipur, India and it was further authenticated by the Botanical Survey of India, Shillong, Meghalaya, India. The matured rhizomes were cleaned shade dried, their skin removed and chopped into thin slices for easy and quick drying. The dried rhizomes were powered using an electrical grinder at room temperature. A known amount of powdered rhizome of *C. gigantea* was sequentially extracted in petroleum ether, chloroform, ethanol and distilled water in order of increasing polarity using a Soxhlet apparatus. Each extracts, except petroleum ether was concentrated in vacuo and stored at -70°C until further use. The ethanol extract was used for the entire study and henceforth it will be called as CGE.

2.3. Drug/s dissolution

Doxorubicin was freshly dissolved in MEM and the ethanol extract of *Colocasia gigantea* were freshly dissolved in distilled water and diluted and filter sterilized immediately before use.

2.4. Cell culture

HeLa and V79 cells were procured from the National Centre for Cell Science, Pune, India. The cells were grown in 25 cm^2 culture flasks (Corning Inc., Corning, NY, USA) containing 5 ml Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum, 1% L-glutamine and 50 µg/ml gentamicin. The cells were incubated at 37°C in an atmosphere of 5% CO_2 in humidified air in a CO_2 incubator (Eppendorf AG, Hamburg, Germany).

2.5. Experimental Design

A fixed amount of cells were seeded int 96 well plates or culture vessels that were divided into the different groups depending on the experimental protocol:

2.5.1.Determination of Cytotoxicity

2.5.1.1. MTT assay

***MEM group*:** The cells of this group served as negative control group and did not receive any treatment.

CGE group: This group of cells was treated with different concentrations of CGE.

DOX group: The cell cultures of this group were treated with5, 10 and 20 µg/ml of doxorubicin (DOX) and served as positive control.

The cytotoxic effects of different concentrations of ethanol extract of *Colocasia gigantea* was studied by MTT assay in HeLa and V79cells as described by Mosmann (1983). Usually 10^4 cells were seeded into 96 well plates (HiMedia, Mumbai, India) in 100 µl minimum essential medium (MEM). The microplates were kept at 37ºC in a CO_2 incubator in an atmosphere of 5% CO_2 in 95 % humidified air and the cells were allowed to attach for 24 h. Next day different concentrations of CGE or doxorubicin were added into each well of the microplates and incubated in the CO_2 incubator. After 48 hours, MTT was added into each well and the microplates were incubated for another 2 hours. The drug containing media were removed and the insoluble purple formazan formed was dissolved using lysis buffer and incubated once again for 4 hours after which the absorbance was recorded at 560 nm using a microplate reader (Spectramax M2, Molecular Devices, Sunnyvale, CA, USA). The cytotoxicity was calculated using the formula: Control-Treatment/Control X 100.

2.5.2. Determination of optimum exposure time for cytotoxicity

A separate experiment was conducted to study the effect of treatment duration of CGE on the cytotoxicity, where grouping and other conditions were essentially similar to that described above except that the cells were exposed to CGE for 2, 4 and 6 h and the cytotoxicity was determined by MTT assay as described above.

2.5.3. Determination of anticancer activity

Another experiment was performed to evaluate the anticancer activity of CGE, where grouping and other conditions were similar to that described in the

experimental design. The anticancer activity of CGE was determined by inoculating 10^6 exponentially growing HeLa cells into several culture flasks. The cells were allowed to attach for 24 h and were treated with 100, 200 and 300 µg/ml CGE.

After 2 hours of drug treatment the media were removed and the flasks were washed twice with sterile PBS, and dislodged by trypsin EDTA treatment and the following studies were conducted.

2.5.4. Clonogenic Assay

Clonogenicity of cells was determined by clonogenic assay (Puck and Marku, 1955). Usually 200 HeLa cells were seeded into several individual petridishes containing 5 ml MEM and left undisturbed for colony formation for another 11 days. After the end of day 11 the resultant colonies of cells were stained with 1 % crystal violet in methanol and scored. Plating efficiency (PE) of the cells was determined and surviving fraction (SF) calculated.

PE = (Number of colonies counted x 100) / (Number of cells seeded)

SF = (Number of colonies counted) / (Number of cells seeded) x (mean plating efficiency).

2.6. BIOCHEMICAL ASSAYS

A separate experiment was carried out to estimate the effect of 100, 200 and 300 µg/ml CGE ()on the activities of various antioxidants in HeLa cells at 2, 4 and 6h post drug treatment. The drug containing media were removed; the cells were washed with sterile PBS and displaced using trypsin EDTA treatment. The cells were pelleted and weighed and 5% homogenate of the cells was prepared in PBS (pH 7.4) using ultrasonicator (PCI Analytics Pvt. Ltd., Mumbai, India). The following assays were carried out:

2.6.1. Glutathione estimation

Glutathione estimation was carried out as described earlier (Moron *et al.,* 1979). Briefly, 1.8 ml of 0.2M Na_2HPO_4 was mixed with 40 µl 10 mM DTNB and 160 µl of cell homogenate. The mixture was allowed to stand for 2 minutes at room temperature and the absorbance was read against the blank at 412 nm in a UV-VIS Biospectrophotometer (Eppendorf India Limited, Kolkata, India).The blank consisted of distilled water instead of cell homogenate.

2.6.2. Glutathione - S – transferase estimation

Glutathione-s-transferase activity was estimated by the method of Habig *et al.,* 1974). Briefly, 0.5 ml of 0.1 M phosphate buffer pH 6.5, 0.1ml of 20mM CDNB, and 8.8 ml distilled water were incubated at 37°C for 10 min. After incubation, 0.5 ml of 20 mM GSH and 0.1 ml of cell homogenate were added. The absorbance was read at 340 nm at 1 min intervals for 6 minutes in UV-VIS Biospectrophotometer.

2.6.3. Catalase

The method of Aebi (1984) was followed for catalase estimation. Briefly, 20 µl of sample was diluted with 1.98 ml of 50 mM phosphate buffer (pH 7.0)in a 3 ml cuvette and the reaction (maintained at 20ºC) was started by adding 1 ml of 30 mM H_2O_2. The decrease in absorbance was monitored at 240 nm for 60 seconds in a UV-VIS Biospectrophotometer.

2.6.4. Estimation of lipid peroxidation

Lipid peroxidation (LOO) assay was carried out by the method of Buege and Aust (1978). Briefly, 1 ml of cell homogenate was mixed with 2 ml of TCA-TBA-HCl reagent and mixed thoroughly. The reaction mixture was heated in a boiling water bath for 15 minutes, cooled immediately to room temperature, centrifuged at

1000 rpm for 10 min and supernatant was collected and its absorbance was read at 535 nm against blank in a UV-VIS Biospectrophotometer.

3. STATISTICAL ANALYSES

The statistical analyses were performed using Origin Pro 8. All the results are expressed as Mean ± Standard Error of the Mean (SEM). Experimental data were analyzed by one way ANOVA followed by Tukey's test for multiple comparisons for different parameters between the groups. A P value of < 0.05 was considered as significant. The experiments were repeated for confirmation and since the difference between the original and repeat experiments was statistically non-significant the data of both the experiments were combined and presented in tables and figures.

4. RESULTS

The results are expressed in table 1-7 and figure 1-9 as Mean ± Standard Error of the Mean (SEM).

4.1. Determination of Cytotoxicity

Treatment of HeLa and V79 cells with different concentrations of CGE caused a concentration dependent increase in the cytotoxicity and the maximum cytotoxic effect was recorded for the highest concentrations of 300µg/ml CGE (Table 1). The cytotoxicity between of 200 and 300µg/ml CGE was not statistically significant the former was chosen for other experiments (Figure 1). Similarly, CGE induced maximum cytotoxicity at 200µg/ml in V79 cells (Figure 2). The positive control DOX also showed a similar pattern (Figure 1-2).

Chapter 4

Determination of treatment duration

The optimum CGE treatment duration for cytotoxic effect was also evaluated by MTT assay at 2, 4 and 6 hours. The CGE treatment resulted in a time dependent increase in the cytotoxicity in HeLa and V79 cells and maximum cytotoxic effect was observed in the cells treated with CGE for h respectively (Figure 3-4). However, this increase was not statistically significant hence 2 h treatment duration was selected for further experiments.

Clonogenic Assay

Treatment of HeLa cells with different concentrations of CGE caused a concentration dependent decline in the clonogenicity of cells (Table 3 and Figure 5). A maximum decline in the clonogenicity was observed for 300 µg/ml CGE, where the surviving fraction of HeLa cell reached a nadir (0.22) less than half of 200 µg/ml (Figure 5).

Glutathione

Treatment of HeLa cells with different concentrations of CGE caused a concentration dependent but significant depletion in glutathione contents at all the post-treatment times (Table 4 and Figure 6). The GSH concentration also declined in a time dependent manner and maximum decline was observed at 6 h post treatment (Figure 6). The concentration of glutathione also declined in a similar as DOX treated group (Table 4 and Figure 6).

Glutathione-s-transferases

GST actvity declined in a concentratoin depenent manner and it was significant lower than the MEM treated group ((Table 5). The acivity of GST also reducted with time in the HeLa cells treated with diffrent oncentrations of CGE and a greatest decline was observed at 6 h post-treatment and for 300µg/ml (Figure 7).

Catalase

The catalase activity also alleviated with increasing CGE concentration and there was significant reduction in the catalase activity at all post-treatment assay time when compared to MEM treatment (Table 6). The analysis of catalase activity with time showed a time dependent decline in the catalase activity for all CGE concentrations and it was lowest at 6 h post treatment (Figure 8). The DOX treatment also resulted in an identical decline in catalase activity at all post treatment times (Table 6 and Figure 8).

Lipid Peroxidation

The treatment of HeLa cells with different concentrations of CGE induced LOO efficiently as indicated by a concentration dependent rise in the LOO at all post-treatment times (Figure 9). This increase in LOO was significantly higher and it was at least 6 folds higher at 6 h post treatment in CGE treated group (Table 7). The maximum LOO was detected at 6 h post treatment in all the groups (Figure 9). The DOX treatment also showed a pattern similar to that of CGE treatment (Figure 9).

Chapter 4

DISCUSSION

The adverse effects induced by modern chemotherapeutic regimens and development of therapy resistance is the major stumbling block for successful treatment of tumors (Raji, 2005; MacDonald, 2009; Houseman *et al.,* 2014; Lee *et al.,* 2017). The other disadvantage of systemic chemotherapy is induction of second malignancies due to genomic damge in the normal cells (Morton *et al.,* 2014). Therefore screening of newer paradigms that do not trigger the development of adverse effects and second malignancies are of crucial importance. The natural products and plants can provide the opportunity to develop non-toxic and effective drug molecules to treat cancer. Therefore the present study was undertaken to evaluate the anticancer potential of *Colocasia gigantea* in cultured HeLa cells.

The cytotoxic effect of any drug candidate/s can be ascertained by MTT assay, which is a rapid and standard technique to determine the cytotoxicity of any drug/treatment. The viable cells or metabolically active cells are able to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT into formazan by the action of mitochondrial succinate dehydrogenase enzyme and the level of activity is a measure of the viability of the cells. The weaker the color formed, the more are the dead cells. MTT assay has been used to test cytotoxicity of numerous drugs in various cell lines since its discovery (Mossmann, 1983). The treatment of HeLa and V79 cells with ethanol extract of *Colocasia gigantea* caused a concentration dependent rise in the cytotoxicity. There are no reports regarding the evaluation of cytotoxicity of ethanol extract of *Colocasia gigantea.* However MTT assay has been used to investigate the cytotoxic effects of other plants in vitro (Booth et al., 2012;

Nguta *et al.,* 2016; Kuete *et al.,* 2017). The cytotoxic effect of CGE was further confirmed by clonogenic assay, which is long term assay to study the toxicity of any agent. The CGE treatment led to a concentration dependent decline in the clonogenicity of HeLa cells. The cytotoxic effect of ethanol extract of *Colocasia gigantea* has not been studied yet. However the other medicinal plants like *Tinospora cordifolia*, and *Aphanmixis polystchya* and synthetic molecules including doxorubicin, daunorubicin and cytarabinehave been reported to alleviate the clonognonic potential of cultured cells earlier (Williams *et al.,* 2010; Jagetia and Rao, 2011, 2015; Jagetia and Venkatesh, 2015, Jagetia and Venkatesha, 2016).

Almost all cancer cells are at increased oxidative stress, which may be essential for progression and development of tumor. The tumors also express high level of antioxidants to balance the increased oxidative stress and this increased antioxidant level is linked with the survival advantage in the tumor cells and also it helps to develop resistance to chemotherapy (Liou and Storz, 2010; Gill *et al.,* 2016). The excess oxidative stress induced by chemotherapeutic drugs is responsible for cell death as it stimulates various mechanism of cell death including non apoptotic form of cell death (Liu and Wang, 2015). Lipid peroxidation is a measure of oxidative stress as the products of lipid peroxidation damage the important macromolecules like proteins and nucleic acid which final lead to death of the cell (Barrera, 2012; Zhong and Yin, 2015; Gaschler and Stockwell, 2017). The CGE increased the oxidative stress in a concentration and time dependent manner and this may be the reason for effective cell killing in the present study. Most of the chemotherapeutic agents kill neoplastic cells by increasing oxidative stress in the tumor cells (Conklin,

2004; Gorrini *et al.,* 2013). Malondialdehyde (MDA) is a major product of lipid peroxidation (Rice-Evans and Burdon, 1993). MDA has the ability to react with nucleic acid bases and form adducts to dG, dA, and dC (Marnett, 2002). Lipid peroxidation has been implicated in the pathogenesis of a number of diseases including cancer due to its ability to damage DNA and subsequent mutations in the tumor suppressor genes (Cejas *et al.,* 2004; Zhong and Yin, 2015). This property of lipid peroxidation may be responsible for killing tumor cells in the present study. The glutathione is the most abundant non-protein intracellular antioxidant that has diverse role in numerous physiological processes (Lushchak, 2012). the increase in glutathione has been implicated in tumor progression and resistance to chemotherapy and reduced glutathione levels have been reported to kill tumor cells more effectively (Circu and Aw, 2012; Franco, and Cidlowski, 2012; Traverso *et al.,* 2013; Rocha *et al.,* 2014; Ramsay and Dilda, 2014). A similar mechanism seems to operational in the present study where the treatment of HeLa cells with CGE has reduced the GSH concentration in a time and concentration dependent manner. The enzyme GST catalyze the nucleophilic attack of glutathione (GSH) on electrophilic substrates by binding with glutathione on its hydrophilic G-site and its adjacent H-site with the electrophilic substrates to bring them in a close proximity. They also activate the sulfhydryl group on GSH, thereby allowing for nucleophilic attack of GSH on the electrophilic substrate (Armstrong, 1997). Elevated levels of GST in tumor cells are associated with increased resistance to apoptosis (McIlwain *et al.,* 2006; Zeng *et al.,* 2014). The CGE reduced the GST activity in a concentration and time dependent fashion, that may have induced effective killing of HeLa cells. Various GST inhibitors have been shown to modulate drug resistance by sensitizing tumor cells to

anticancer drugs (Townsend and Tew, 2003; Laborde, 2010).. Catalase or oxidoreductase is present in all organisms and it detoxify H_2O_2 into water and oxygen and it is also involved in various other processes. High levels of catalase have been reported in patients with lung cancer, whereas decreased levels of catalase were indicated in breast cancer, head and neck cancer, gynaecological cancer, lymphoma, prostate cancer and urological cancer (Kodydková *et al.,* 2014). The over expression of catalase has been reported to reduce the apoptosis in tumor cells after chemotherapy (Bechtel and Bauer, 2009). The treatment of HeLa cells with CGE depleted the activity of catalase in concentration and time dependent manner, which would killed the HeLa cells effectively.

The mechanisms of cell killing by CGE are mostly not understood. However present study makes it very clear that CGE administration has increased the lipid peroxidation more than 6 fold thereby leading to a rise in the oxidative stress, which would have damaged the cellular DNA, other biomolecules and membranes killing the cells. The alleviated levels of GST, catalase and GSH would have further increased the oxidative stress and added insult to injury killing the HeLa cells effectively. The cancer and cancer cell lines over express the COX-II and nuclear transcription factors NF-κB and Nrf2 and they are also involved in resistance to tumor therapy ((Sobolewski *et al.,* 2010; Lu and Stark, 2015; Choi and Kwak, 2016). The suppression of transcriptional activation of these genes by CGE may have played an important role in effectively killing the cells. The induction of apoptosis and activation of p53 and related proteins may have also contributed their share in bringing cell death.

Chapter 4

CONCLUSIONS

The present study clearly demonstrates the cell killing ability of CGE and the cell killing may be due to the increased LOO, accompanied by a decline in the GSH, GST and catalase, that would have increased the oxidative stress that may have triggered the DNA, protein and membrane damage killing the cells effectively. CGE may have also suppressed the activation of COX-II, NF-κB and Nrf2 elements that may have induced apoptotic cell death. The over expression of p53 and related proteins may have also contributed to cee death in the present study.

The present study demonstrates that *Colocasia gigantea* exerted cytotoxic effect in a concentration dependent manner and the cytotoxic effect may be due to increased lipid peroxidation accompanied by a decrease in GSH, GST and catalase.

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Table 1: Effect of different concentrations on the cytotoxic effects of ethanol extract of *Colocasia gigantea* (CGE) in various cell lines by conventional MTT assay parantheses indicates the dose for Doxorubicin (DOX). The results were determined as percentage (%) cytotoxicity and expressed as Mean ± SEM, n=5, p<0.05.

Dose(µg/ml)		Cell Line	Cytotoxicity
Doxorubicin	5	Hela	57.13 ± 0.07
	10		62.56 ± 2.56
	20		83.01 ± 0.19
CGE	12.5		25.80 ± 2.52
	25		31.81 ± 1.18
	50		39.11 ± 1.33
	100		45.48 ± 1.37
	200		52.74 ± 1.57
	300		55.84 ± 1.69
	400		53.77 ± 0.57
Doxorubicin	5	V79	72.04 ± 0.36
	10		67.71 ± 1.40
	20		65.49 ± 1.33
CGE	12.5		48.43 ± 0.44
	25		43.34 ± 4.57
	50		31.49 ± 3.17
	100		45.02 ± 2.72
	200		53.85 ± 2.19
	300		52.61 ± 1.72
	400		51.31 ± 1.61

Standard error of the mean (SEM).
Colocasia gigantea (CGE).
Doxorubicin (DOX).

Table 2: Effect of different exposure time on the cytotoxic effects of ethanol extract of *Colocasia gigantea* (CGE) and Doxorubicin (DOX) in various cell lines by MTT assay at different post treatment time. The data are expressed as Mean ± SEM, n=5, p<0.05.

Treatment (µg/ml)	Cell line	Cytotoxicity (Percent ±SEM)		
		Post Treatment Time (hour)		
		2	4	6
Doxorubicin 5	HeLa	8.63 ± 1.08	10.56 ± 0.97	17.70 ± 1.06
10		11.64 ± 0.95	13.27 ± 1.49	21.59 ± 1.27
20		18.49 ± 2.28	21.54 ± 1.44	27.44 ± 1.91
CGE 100		32.87 ± 1.62	37.23 ± 1.72	46.29 ± 4.06
200		41.78 ± 0.98	50.64 ± 1.40	54.54 ± 4.47
300		42.73 ± 1.95	54.92 ± 1.16	57.23 ± 2.54
Doxorubicin 5	V79	3.28 ± 1.65	5.45 ± 0.91	11.50 ± 0.58
10		10.13 ± 0.71	13.45 ± 0.87	17.54 ± 0.68
20		15.91 ± 1.87	20.94 ± 0.96	27.03 ± 0.77
CGE 100		40.39 ± 0.81	41.33 ± 0.90	41.95 ± 0.16
200		43.82 ± 1.40	50.41 ± 0.52	50.47 ± 2.12
300		48.96 ± 1.54	55.79 ± 0.64	57.40 ± 0.37

Standard error of the mean (SEM).

Colocasia gigantea (CGE).

Doxorubicin (DOX).

Table 3: Effect of different concentrations of the ethanol extract of *Colocasia gigantea* (CGE) and Doxorubucin (DOX) treatment on the survival of HeLa cells. The result are expressed as Mean ± SEM, n=3.

Treatment	Surviving Fraction
MEM	1.005 ± 0.003
DOX 5 µg/ml	0.472 ± 0.014*
DOX 10 µg/ml	0.286 ± 0.007*
DOX 20 µg/ml	0.255 ± 0.01*
CGE 100 µg/ml	0.692 ± 0.009*
CGE 200 µg/ml	0.479 ± 0.007*
CGE 300 µg/ml	0.377 ± 0.004*

**P<0.01 when treatment groups are compared to control group (MEM).*
No symbol= no significance.
Standard error of the mean (SEM).

Table 4: Alterations in the Glutathione content of HeLa cells induced by different concentrations of *Colocasia gigantea* and Doxorubicin. The results were determined as µmol/ mg protein and expressed as Mean ±SEM, n=5.

Post Treatment Time (h)	MEM	Treatment (µg/ml)					
		Colocasia gigantea (CGE)			Doxorubicin (DOX)		
		100	200	300	5	10	20
2	2.978 ± 0.03	2.42 ± 0.01 *	1.23 ± 0.02 *	1.01 ± 0.01 *	1.68 ± 0.02 *	1.03 ± 0.02 *	0.88 ± 0.007 *
4	3.01 ± 0.079	2.03 ± 0.006 *	1.58 ± 0.005 *	1.03 ± 0.007 *	1.76 ± 0.04 *	1.58 ± 0.02 *	1.101 ± 0.012 *
6	2.665 ± 0.017	1.67 ± 0.02 *	1.57 ± 0.05 *	1.5 ± 0.007 *	1.99 ± 0.005 *	1.105 ± 0.03 *	0.67 ± 0.007 *

**p<0.01 when treatment are compared to concurrent control (MEM) group.*
Standard error of the mean (SEM).
Minimum essential media (MEM).
No symbol = no signigicant difference.

Table 5: Alterations in the GST activity of HeLa cells treated with different concentrations of *Colocasia gigantea* and doxorubicin. The results were determined as Unit/ mg protein and expressed as Mean ±SEM, n=5.

Post Treatment Time (h)	MEM	Treatment (µg/ml)					
		Colocasia gigantea (CGE)			Doxorubicin (DOX)		
		100	200	300	5	10	20
2	0.18 ± 0.02	0.14 ± 0.01 #	0.11 ± 0.06 *	0.09 ± 0.01 *	0.14 ± 0.05 *	0.07 ± 0.03 *	0.04 ± 0.02 *
4	0.17 ± 0.02	0.13 ± 001 #	0.09 ± 0.03 #	0.08 ± 0.01 *	0.14 ± 0.03 #	0.08 ± 0.02 *	0.05 ± 0.019 *
6	0.17 ± 0.01	0.09 ± 0.01 #	0.07 ± 0.03 *	0.04 ± 0.02 *	0.13 ± 0.02 *	0.09 ± 0.02 *	0.05 ± 0.012 *

**P<0.01, # P<0.05 when treatment are compared to concurrent control (MEM) group.*

No symbol= no significant difference.

Standard error of the mean (SEM).

Table 6: Alterations in the catalase activity of HeLa cells treated with different concentrations of *Colocasia gigantea* extract (CGE) and doxorubicin (DOX). The results were determined as Unit/ mg protein and expressed as Mean ±SEM, n=5.

Post Treatment Time (h)	MEM	Treatment (µg/ml)					
		Colocasia gigantea (CGE)			Doxorubicin (DOX)		
		100	200	300	5	10	20
2	7.66 ± 0.72	5.18 ± 1.2	4.76 ± 0.29	3.76 ± 0.2 *	5.8 ± 0.1	3.87 ± 0.1 *	1.9 ± 0.1 *
4	8.65 ± 0.77	4.76 ± 0.2 *	3.97 ± 0.19 *	3.54 ± 0.4 *	5.56 ± 0.56 *	31.65 ± 0.19 *	1.43 ± 0.1 *
6	8.54 ± 1.02	3.97 ± 0.19 *	3.2 ± 0.2 *	2.65 ± 0.39 *	5.87 ± 0.1 *	4.08 ± 0.1 *	2.87 ± 0.1 *

**p<0.01 when treatment are compared concurrent control (MEM) group.*

Standard error of the mean (SEM).

No symbol= no significant difference.

Table 7: Alterations in the Lipid peroxide level in the HeLa cells treated with different concentrations of *Colocasia gigantea* extract (CGE) and doxorubicin (DOX). The results were determined as nmol/ mg protein and expressed as Mean ±SEM, n=5.

Post Treatment Time (h)	MEM	Treatment (µg/ml)					
		Colocasia gigantea (CGE)			Doxorubicin (DOX)		
		100	200	300	5	10	20
2	5.75 ± 0.82	19.77 ± 1.82*	21.08 ± 1.23*	31.31 ± 1.25*	21.43 ± 2.17*	27.86 ± 1.35*	31.55± 1.22*
4	6.71 ± 0.54	25.72 ± 2.71*	34.52 ± 1.52*	35.64 ± 1.26*	28.5 ± 2.53*	40.21 ± 1.81*	45.33 ± 2.15*
6	5.62 ± 0.72	32.13 ± 1.32*	48.63 ± 1.23*	59.68 ± 1.76I*	37.18 ± 1.87*	48.37 ± 2.35*	53.98± 2.45*

**p<0.01 when treatment groups are compared to concurrent control.*

No symbol= No significance.

Standard error of the mean (SEM).

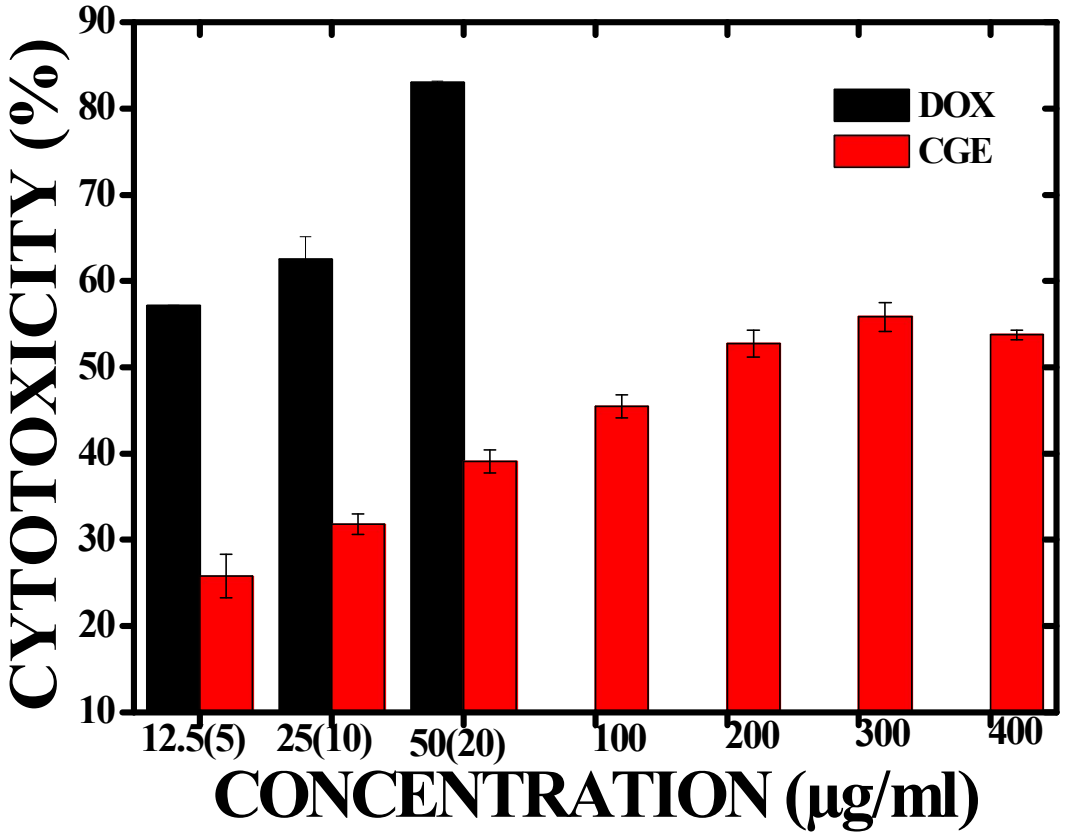

Figure 1: The cytotoxic effect of different concentrations of ethanol extract of *Colocasia gigantea* and Doxorubicin on HeLa cells assessed by dose dependent MTT assay. CGE- Ethanol extract of *Colocasia gigantea,* DOX- Doxorubicin. Figures in brackets indicate concentration of doxorubicin.
The data represent Mean±SEM, N=5.

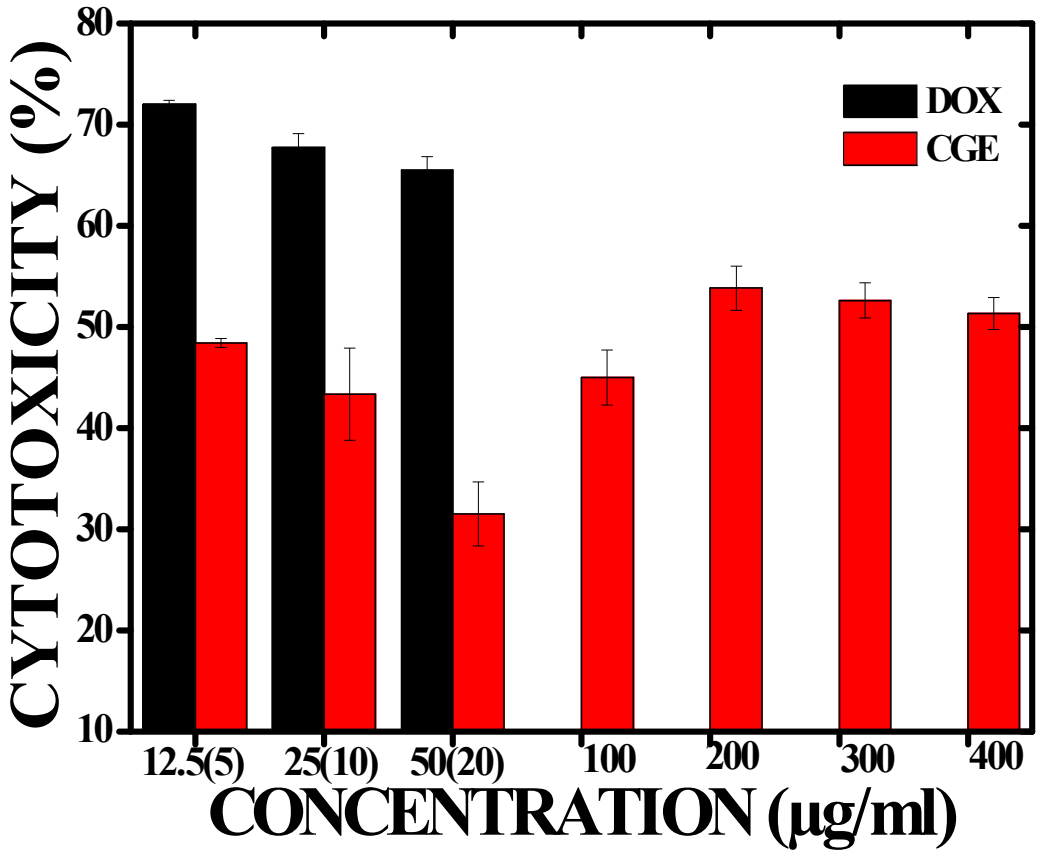

Figure 2: The cytotoxic effect of different concentrations of ethanol extract of *Colocasia gigantea* and Doxorubicin on V79 cells assessed by dose dependent MTT assay. CGE- Ethanol extract of *Colocasia gigantea,* DOX- Doxorubicin. Figures in brackets indicate concentration of doxorubicin.
The data represent Mean±SEM, N=5.

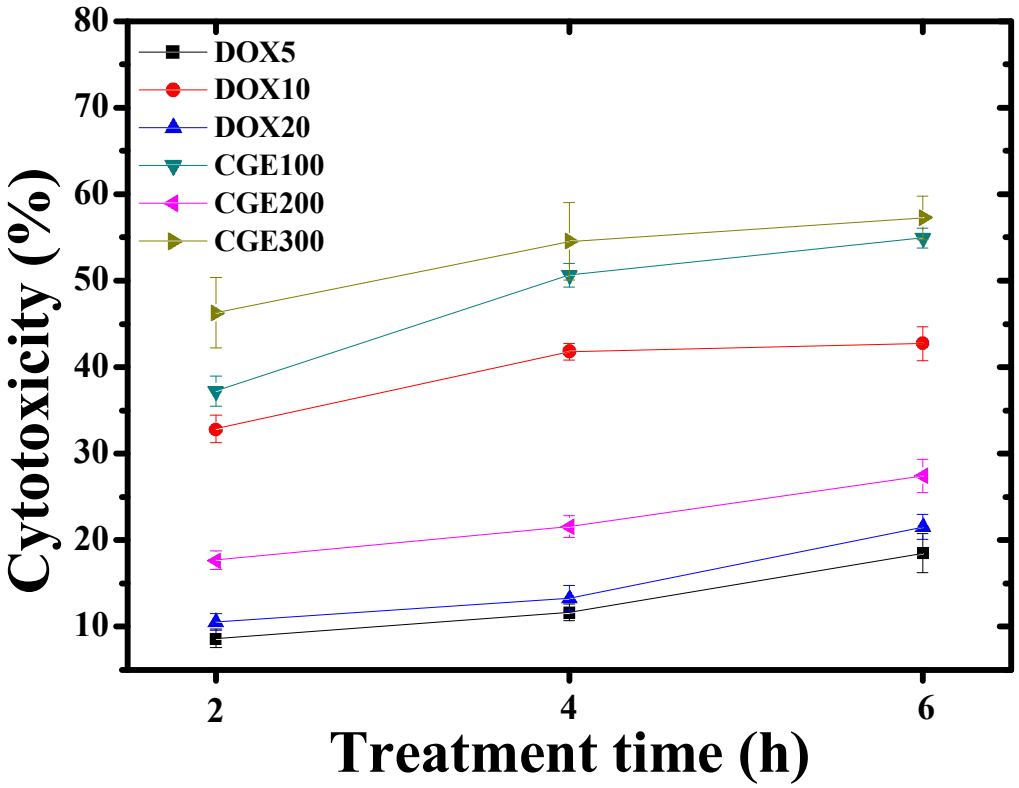

Figure 3: The effect of different concentration of the ethanol extract of *Colocasia gigantea* and Doxorubicin on HeLa cells determined by Time dependent MTT assay. Ethanol extract of *Colocasia gigantea* (CGE), Doxorubicin (DOX).

The data represent Mean ± SEM, N=5.

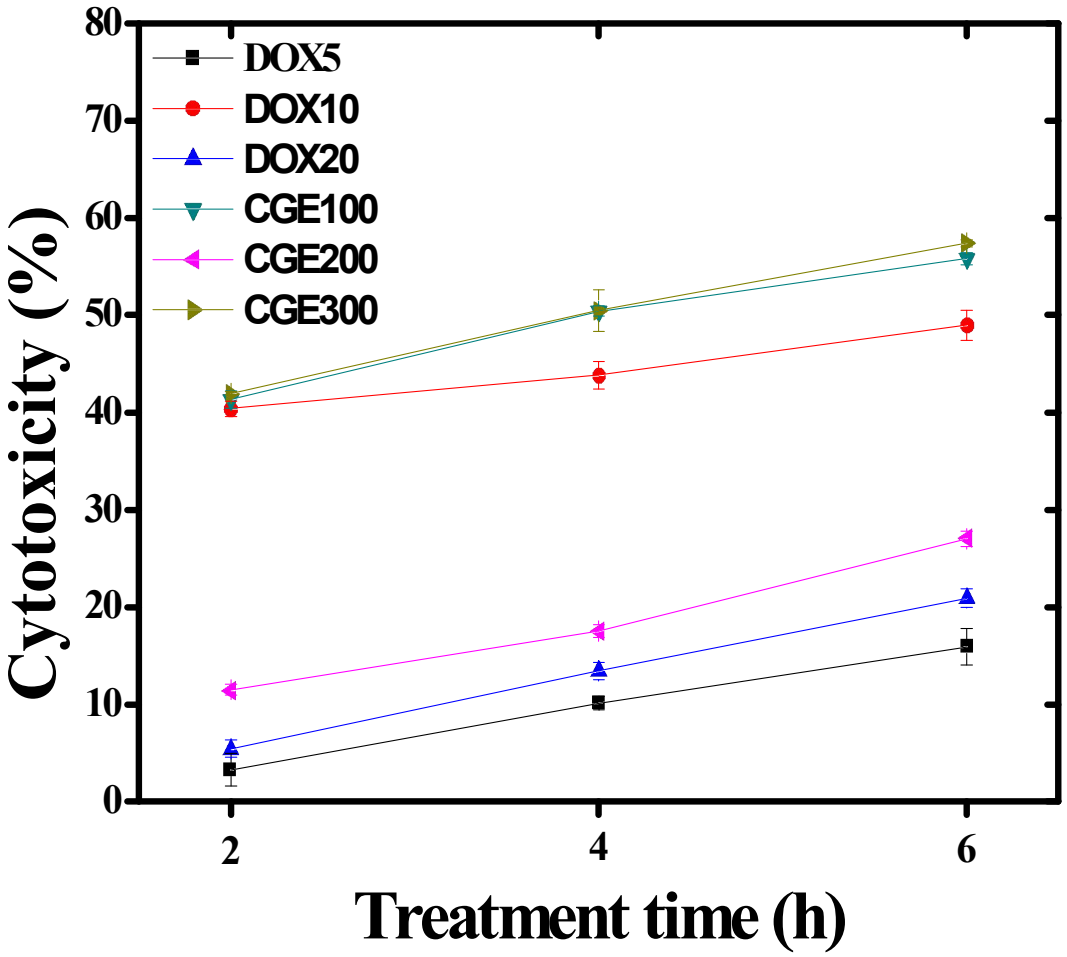

Figure 4: The effect of different concentration of the ethanol extract of *Colocasia gigantea* and Doxorubicin on V79 cells determined by Time dependent MTT assay. Ethanol extract of *Colocasia gigantea* (CGE), Doxorubicin (DOX).

The data represent Mean ± SEM, N=5.

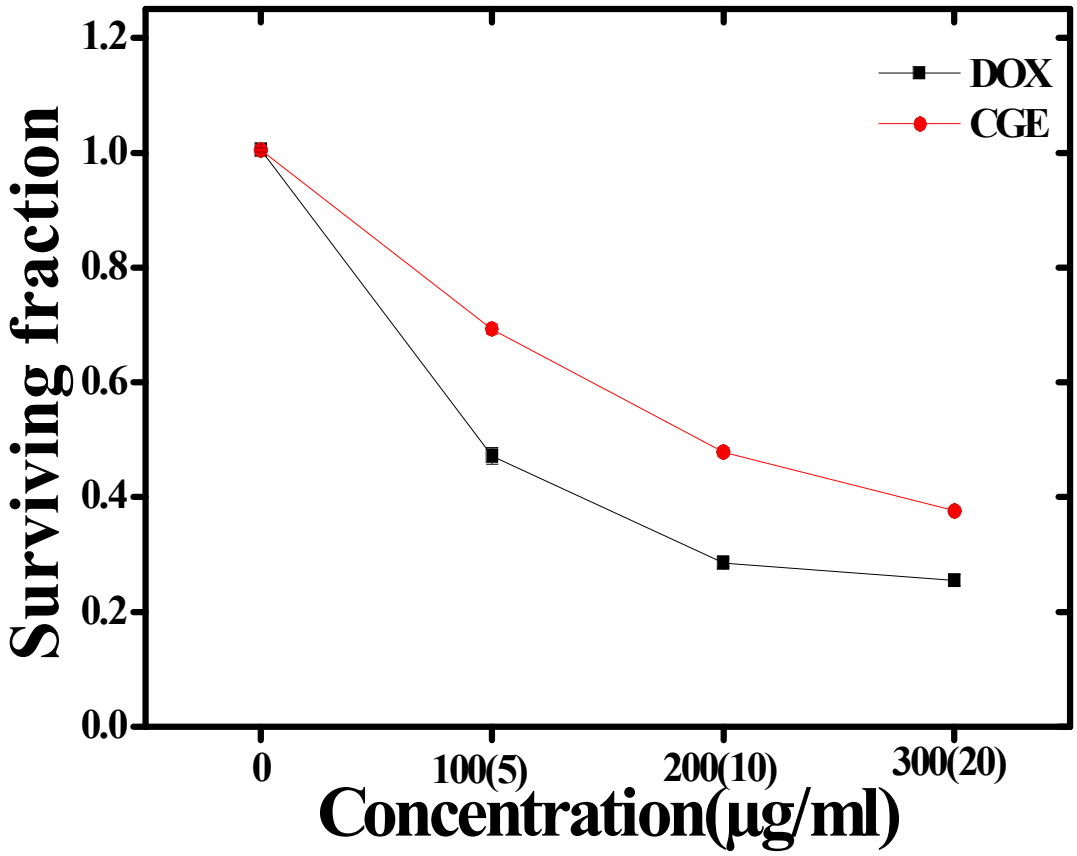

Figure 5: Effect of different concentrations of the ethanol extract of *Colocasia gigantea* (CGE) and Doxorubucin (DOX) treatment on the survival of HeLa cells. Figures in brackets on X-axis indicate concentration of Doxorubicin. The results are expressed as Mean ± SEM, N=3.

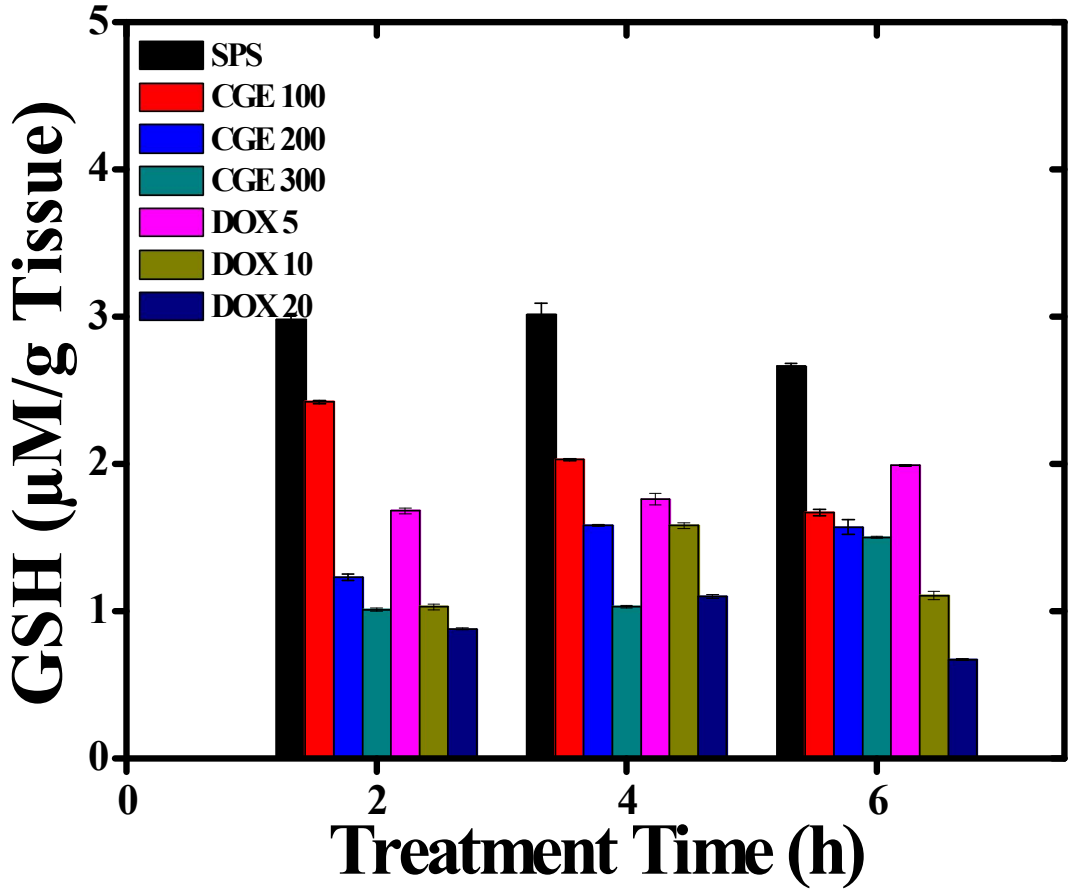

Figure 6: Alteration in the GSH activity of cultured HeLa cells treated with different concentrations of CGE and DOX. Minimum essential media (MEM), Ethanol extract of *Colocasia gigantea* (CGE), Doxorubicin (DOX). The data represent Mean±SEM, N=5. *p<0.01 when the treatment group are compared to MEM group. Standard error of the mean (SEM).

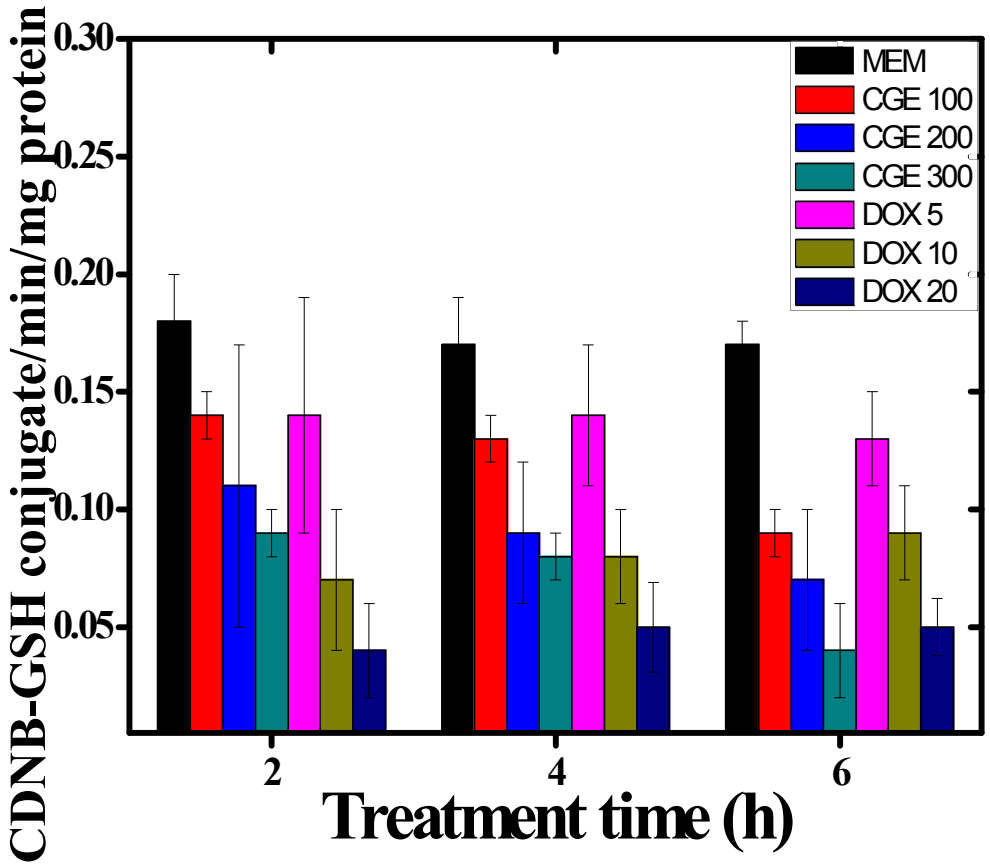

Figure 7: Alteration in the GST activity of cultured HeLa cells treated with different concentrations of CGE and DOX. Minimum essential media (MEM), Ethanol extract of *Colocasia gigantea* (CGE), Doxorubicin (DOX). The data represent Mean±SEM, N=5. *p<0.01 when the treatment group are compared to MEM group. Standard error of the mean (SEM).

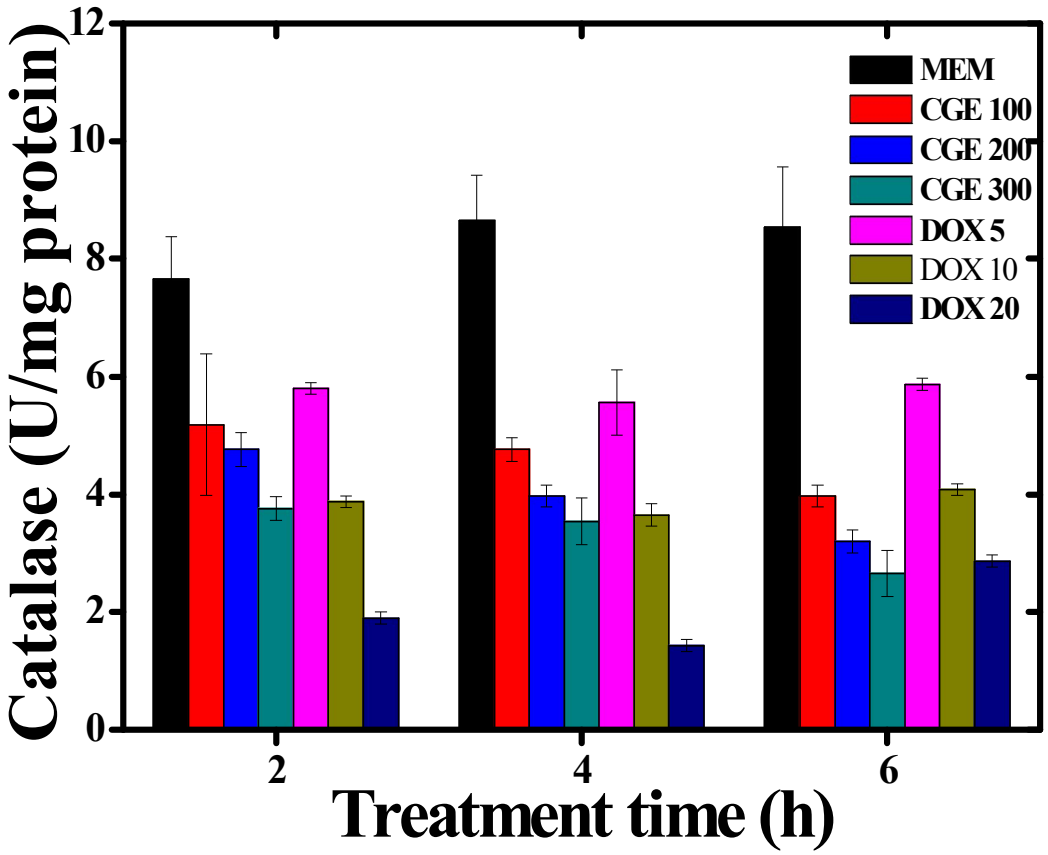

Figure 8: Alteration in the Catalase activity of cultured HeLa cells treated with different concentrations of CGE and DOX. Minimum essential media (MEM), Ethanol extract of *Colocasia gigantea* (CGE), Doxorubicin (DOX). The data represent Mean±SEM, N=5. *p<0.01 when the treatment group are compared to MEM group. Standard error of the mean (SEM).

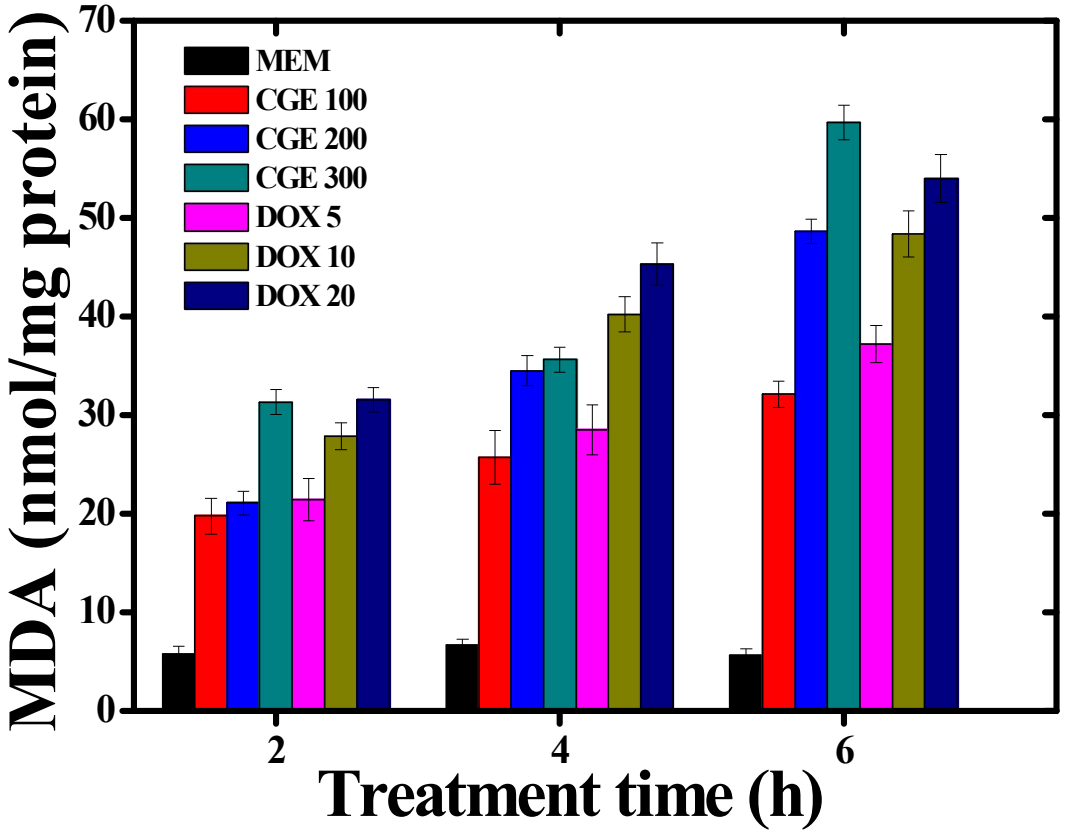

Figure 9: Alteration in the Lipid peroxidation activity of cultured HeLa cells treated with different concentrations of CGE and DOX. Minimum essential media (MEM), Ethanol extract of *Colocasia gigantea* (CGE), Doxorubicin (DOX). The data represent Mean±SEM, N=5. *p<0.01 when the treatment group are compared to MEM group. Standard error of the mean (SEM).

CHAPTER 5

ANTITUMOR ACTIVITY OF THE DIFFERENT EXTRACTS OF *COLOCASIA GIGANTEA* (BLUME) HOOK. *f.* IN DALTON'S LYMPHOMA TRANSPLANTED SWISS ALBINO MICE

Chapter 5

Abstract

The acute toxicity was determined in normal non-tumour bearing mice administered with different doses of various extracts of Colocasia gigantea orally or and intraperitoneally. The oral administration of chloroform, ethanol and aqueous extracts of Colocasia gigantea were non-toxic upto 2 g/kgbody weight. The intraperitoneal administration of different extracts of Colocasia gigantea exhibited toxic effect and the LD50 for ethanol extract was found to be 0.2 g/kgb.wt. whereas it was 0.15 g/kgb.wt. for chloroform and aqueous extracts, respectively. The determination of anticancer activity by intraperitoneal administration of 0, 50, 75, 100, 125, 150, 175, 200 or 225 mg/kgb.wt.of ethanol extract to Dalton's lymphoma transplanted mice resulted in a dose dependent rise in tumour regression and increase in the average survival as well as median survival time. The maximum tumour free survivors were observed at 200 mg/kgb.wt. of ethanol extract and further experiments were carried out using this dose. The evaluation of micronuclei showed that the ethanol extract Colocasia gigantea increased the frequency of micronucleated mononucleate cells as well as micronucleated binucleate cells in a time dependent manner and their frequencies were maximum at 36 h post-treatment. Similarly ethanol extract Colocasia gigantea increased the apoptosis index also increased in a time dependent manner and the highest apoptosis was observed at 36 h post treatment. The biochemical studies revealed a significant decline in the glutathione, glutathione-s-transferase, superoxide dismutase and catalase accompanied by elevated lipid peroxidation. The safety of ethanol extract of 200mg Colocasia gigantea was ascertained by

evaluating aspartic acid transaminase, and alanine aminotransferase, creatinine and uric acid at different post treatment times in Dalton's lymphoma bearing mice liver and kidney. These parameters did not show any significant alteration and they were within the normal range.The ethanol extract of Colocasia gigantea did not show any toxicity orally however, intraperitoneal administration did exert toxic effects and it also induced anticancer activity in tumour cells by increasing tumour free survivors. The cytotoxic effect of ethanol extract may be due to induction of DNA damage in the form of micronuclei and apoptosis and reduction in glutathione, glutathione-s-transferase, superoxide dismutase and catalase accompanied by a rise in the lipid peroxidation.

Chapter 5

1. INTRODUCTION

The cardiac diseases are the leading cause of death and cancer is the second largest killer disease globally. Despite development of various modern treatment modalities, the mortality rates, especially for solid tumors remains a major cause of concern. It is also projected that mortality rates due to cancer may out number cardiovascular diseases not in too distant in future in developed world (Siegel *et al.,* 2017). The chemotherapy is one of the important modalities to treat cancer and the term "chemotherapy" was introduced by Paul Ehrlich in the early nineteen century who defined chemotherapy as the use of chemicals to treat diseases (Williams, 2009). However, it became associated with cancer treatment in 1943 since then treatment of cancer with chemicals is synonymous with chemotherapy (DeVita, and Chu, 2008). Several synthetic chemicals find their use in the cancer treatment and role of natural products in cancer treatment was realized when alkaloids isolated from *Catharanthus roseus* were found to be useful in the treatment of hematologic malignancies (Johnson *et al.,* 1963; Creasey, 1974; Noble, 1990). Thereafter several other molecules have been isolated from different plants to treat different types of cancers (Newman and Cragg, 2016). Podophyllotoxins separated from the ethanol extract of *Podophyllum peltatum* also showed anticancer activity against a wide range of tumors. The etoposide and teniposide, which are derivatives of podophyllotoxins are in frequent clinical use (Imbert, 1998; Canel *et al.,* 2000; Gordaliza, 2004). Camptothecins are natural product derived cancer chemotherapeutic agents that have found their application in clinics to treat different cancers (Potmesil, 1994; Pizzolato and Saltz, 2003). The taxols are another class of

natural products isolated from plants and are infrequently clinical use to treat a wide range of neoplastic disorders (Rowinsky and Donehower, 1995; Wani and Horwitz, 2014).

The chemical synthesis of natural products further strengthened their use and may still continue to play a significant role in the treatment of cancer in the years to come (Devita and Chu, 2008; Morgan *et al.,* 2014). The currently used chemotherapy for cancer treatment has several side effects and therefore there is a need for better therapy with lesser side effects (Lotfi-Jam *et al.,* 2008). Besides, the high cost as well as lack of effectiveness of the current conventional therapies (chemotherapy and radiation), especially to solid tumors, use of plants for cancer treatment may be alternative and acceptable therapy (Wood-Sheldon *et al.,* 1997). The side effects due to most cancer drug toxicity also act as a driving force to the use of alternative medicine for better cure (Rao *et al.,* 2008). Plants are not only safe for long term therapy but also provide nutrition and reduce the side effects of conventional cancer therapy. The high cost and negative impact of conventional therapy, low-cost and safety of plants has been drawing increased attention towards plants and plant derived products for cancer cure (Jagetia and Venkatesha, 2012). Plants and natural products are still in great demand due to their safety, efficacy and lesser side effects (Thillaivanan and Samraj, 2014; Jagetia, 2017) and about 80% individuals in the developing countries still depend on plants to treat different diseases. There are also reports that 25% of modern drugs are obtained from plants and 70% of the drugs introduced in the United States for the past 25 years have their origin in plants (Kinghorn *et al.,* 2011; Newman and Cragg, 2016). Plants contain many

phytochemicals which work in a synergistic mode of action in such a way that their uses can complement or damage others or neutralize their possible negative effects (Hassan, 2013). The use of multicompounds is preferred over the use of single drug for the treatment of several diseases including cancer, AIDS, diabetes, etc. due to their beneficial effects (Pan *et al.,* 2014). The popularity for use of herbal medicines by general public is due to the belief that botanicals will provide some measure of benefit over and above modern allopathic medical approaches. They are also considered non-toxic or less toxic than the synthetic molecules.

*Colocasia gigantea (*family: Araceae) commonly called giant taro or elephant's ear, is a large, stemless, tuberous, frost-tender perennial herb, which typically grows up to 4-7' tall and has wide and heart-shaped to arrowhead-shaped, conspicuously-veined, downward-pointing, peltate, dull green to gray green leaves (2-4' long) attached to stout, succulent stems. As the common name suggests, each leaf purportedly resembles the ear of an elephant. It is native to valley forests in China and Southeast Asia. In Fiji, the locals make use of either boiled or baked breadfruit or tubers of taro as slices along with roasted pig (Muralidharan, 1992). Along with culinary items of taro it is used as medicine to treat constipation and tuberculosis in Hawaii (Kokua1977). Pressed juice of petiole of taro is highly cystic and is even said to arrest arterial haemorrhage (Drury, 1873). Taro is used as medicine in China (But *et al.*1988) Nutritionally, taro is very similar to tannia. It contains starch 17.5% amylose and the rest as amylopectin. Starch grain is very small and the size ranges from 1-4 μm. It is rich in most of the essential amino acids and hence is considered to be a good leafy vegetable. It is reported that 100 g of taro tuber contains 73.1 g moisture, 3 g protein, 0.1 g fat, 1.7 g minerals, 22.1 g

carbohydrates, 0.04 g calcium, 0.14 g phosphorus, 2.1 mg iron, 80 IU Vitamin B and trace of Vitamin C (Shanmugavelu, 1989). Since not much information is available on *Colocasia gigantea* despite the fact that it is commonly used as a vegetable in India and Southeast Asia as a part of human diet, the present study was undertaken to study the anticancer activity of *Colocasia gigantea* in mice transplanted with Dalton's lymphoma cells.

2. MATERIALS AND METHODS

2.1. Chemicals

Dimethyl sulphoxide (DMSO), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), nitrobluetetrazolium(NBT), phenazinemethosulphate (PMS), reduced glutathione (GSH), triton X-100, ethylenediaminetetra-acetic acid (EDTA), sodium pyruvate, thiobarbituric acid (TBA), ethidium bromide, acridine orange, crystal violet, and cytochalasin B were obtained from Sigma Aldrich Chemical Co. (Kolkata, India). Sodium carbonate (Na_2CO_3), trichloroacetic acid (TCA), potassium chloride (KCl), potassium sodium-tartrate, and hydrogen peroxide (H_2O_2), were procured from SD Fine-Chemicals Ltd., Mumbai, India, whereas sodium hydroxide (NaOH), sodium chloride (NaCl), disodium biphosphate (Na_2HPO_4), sulphuric acid (H_2SO_4), hydrochloric acid (HCl), n-butanol, Tris (hydroxymethyl) aminomethane (Tris buffer) and ammonium oxalate were supplied by Merck India Limited, Mumbai. Phenol-chloroform-isoamyl alcohol (PCI), and reduced nicotinamide adenine dinucleotide (NADH) were purchased from HiMedia, Mumbai, India. Doxorubicin, Getwell Pharmaceuticals, Gurgaon, India.

Chapter 5

2.2. Collection and Preparation of the Extract

The non-infected and matured rhizomes of *Colocasia gigantea* (family-Araceae) were collected from Manipur. The plant was identified by PG Department of Botany, DM college, Imphal-West, Manipur, India and further authenticated by Botanical Survey of India, Shillong, Meghalaya, India. The non-infected and matured rhizomes were cleaned chopped into thin slices to facilitate drying in shade at room temperature. The dried rhizomes were powdered in an electrical grinder at room temperature. A sample of 100 g of powder was extracted sequentially with chloroform, ethanol and water in a Soxhlet apparatus (Suffness and Dorous, 1979). The extract was then concentrated to dryness under reduced pressure and stored at -80 until further use. The chloroform, ethanol and water extracts of *Colocasia gigantea* will be called as CGC, CGE and CGA henceforth.

2.3. Preparation of Drug and mode of administration

The different Chloroform (CGC) ethanol (CGE) and aqueous (CGC) extracts of *Colocasia gigantea* were dissolved in appropriate solvent immediately before use. Doxorubicin was dissolved in sterile physiological saline (SPS) alone. Each animal from each group received different treatments according to body weight intraperitoneally.

2.4. Animal care handling

The animal care and handling were carried out according to the guidelines issued by the World Health Organization, Geneva, Switzerland and the INSA (Indian

National Science Academy, New Delhi, India). Swiss albino mice were bred before use in a controlled environment of temperature (24-25ºC), 50% humidity and light and dark (12 h each) cycle. Usually 5-6 animals were housed in a sterile polypropylene cage containing paddy husk (procured locally) as bedding material. Six to eight weeks old male and female Swiss albino mice weighing 28-35 g were procured from the inbred colony for experimentation. The animals were fed with commercially available food pellets and water ad libitum. The Institutional Animal Ethics Committee of Mizoram University, approved the entire study vide letter no. MZUIAEC16-17-01, Aizawl, India.

2.5. Acute toxicity determination

The acute toxicity of all extracts was determined both orally and intraperitoneally according to guidelines issued by the Organization for Economic Co-operation and Development (OECD). Albino mice selected by random sampling technique (n=10) of both sexes (5 males and 5 females) were used for each dose of individual extract. The animals were fasted for 18 hours (both food and water were withdrawn) prior to oral or intraperitoneal administration of different extracts of *Colocasia gigantea*. The control group received sterile physiological saline (SPS). The animals were weighed before and after fasting to estimate their weight loss. If mortality was observed in 2-3 animals then the dose administered was assigned as toxic dose. If mortality was observed in one animal, then the same dose was repeated again to confirm the toxicity. The LD_{50} for each extracts was calculated using Probit analysis.

Chapter 5

2.6. Tumor Model

Dalton's lymphoma ascites (DLA) tumor, procured from the Department of Zoology, North- Eastern Hills University, Shillong was propagated in 10-12 weeks old mice by serial intraperitoneal transplantation of 1 x 10^6 viable tumor cells in 0.25 ml PBS, pH 7.4 under aseptic conditions.

2.7. Experimental design

Dalton's lymphoma tumor bearing mice were divided into the following groups:

2.7.1. Negative Control groups: Each extracts had its own control group. The negative control group for all the three extracts, viz. chloroform, ethanol and aqueous extracts received SPS alone.

2.7.2. DOX groups: This group of animals was injected with 0.5 mg/kg body weight of doxorubicin, a standard anticancer drug and served as positive control.

2.7.3. CGE groups: This group of animals received 50, 75, 100, 125, 150, 175, 200 or 225 mg/kg body weight of the ethanol extract of *Colocasia gigantea.*

The tumor bearing animals were given treatment once daily 1 day after tumorization for subsequent 9 days (Geran *et al.,* 1972). Each group consisted of ten animals for each dose. The animal survival was monitored daily up to 120 days, since the survival of animals up to 120 days is equivalent to 5 years survival in humans (Nias, 1990). The deaths, if any, of the tumor bearing mice were recorded daily and the survival was determined. The tumor response was assessed by calculating median survival time (MST) and average survival time (AST). The MST

and AST were calculated from the animals dying within 120 days and those surviving beyond 120 days were excluded from the study (Geran *et al.,* 1972). The increase in median life span (% IMLS), increase in average life span (% IALS) and the increase in life span (%ILS) was also calculated using the formulae:

MST= First death + Last death in the group/2

AST= Sum of animals dead on different days/No. of animals

%IMLS= MST of treated mice – MST of control x 100/MST of control

%IALS = AST of treated mice – AST of control x 100/AST of control

% ILS = (T/C x 100) - 100

Where, T is the mean survival days of treated mice and C is that of the control mice.

2.8. Micronucleus Assay

A separate experiment was performed to study the ability of ethanol extract of *Colocasia gigantea* to induce DNA damage in DLA cells .The grouping and other conditions were similar to that described in the experimental design section, except that the animals were injected with 200 mg/kg b. wt. CGE and the micronuclei were assayed ant 12, 24 and 36 h post CGE treatment. The micronuclei were prepared according to the modified method of Fenech and Morley, (1985). Briefly, the DLA cells were aspirated from tumor bearing mice one hour after the last administration of SPS or CGE. The cells were washed twice with sterile PBS and $1X10^6$ cells were inoculated into each well of 6 well sterile plates containing 3 ml of MEM and were allowed to attach for 6 h. Thereafter the cells were treated with 3 µg/ml of

cytochalasin-B to block cytokinesis. The cells were left undisturbed and allowed to grow for different times and terminated at 12, 24 and 36 h after the initiation of the cultures. The media containing cytochalasin-B were removed, the cells were washed twice with PBS, dislodged by trypsin EDTA treatment and pelleted by centrifugation at 1000 rpm for 5 min. The cell pellet was disturbed and cells were kept in mild hypotonic (0.75% ammonium oxalate) at 37°C, centrifuged again and the resultant cell pellet was fixed in Carnoy's fixative 3:1 (Methanol:Acetic acid). The cells were pelleted again by centrifugation, and resuspended in a small volume of fixative. The cells were spread on to pre cleaned coded slides so as to avoid observer's bias. The slides containing cells were stained with 0.25% acridine orange (BDH, England, Gurr Cat.No. 34001 9704640E) in Sorensen's buffer (pH 6.8) and subsequently washed in the buffer to remove excess stain. The Sorensen's buffer mounted slides were observed under a DM-2500 fluorescence microscope (Leica Microsystems, Wetzlar, Germany) equipped with 450–490 nm BP filter set with an excitation at 453 nm using a 40 X N Plan objective. A minimum of one thousand binucleate cells with well-preserved cytoplasm was scored for each culture and usually five cultures were used for each group. A minimum of 5000 cells were scored for each culture for the determination of the frequency of micronucleated binucleate cells (MNBNC). The micronucleated cells were scored according to the criteria of Kirsch-Volders *et al.,* (2003) and Fenech *et al.,* (2003).

2.9. Apoptosis Assay

A separate experiment was conducted to determine whether *Colocasia gigantea* has the ability to enhance apoptosis in Dalton's lymphoma cells. The

grouping and other conditions were essential similar to that described for micronucleus assay. DLA cells were aspirated from tumor bearing mice one hour after the last administration of SPS or CGE. The cells were washed twice with sterile PBS and $1X10^6$ cells were inoculated into each well of 6 well sterile plates containing 3 ml of MEM. The cells were allowed to grow for 12, 24 and 36 h to assess apoptosis. The induction of apoptosis was studied at 12, 14 and 36 h post drug treatment as described earlier (Ribble *et al.,* 2005). The tumor cells were aspirated and washed with ammonium chloride to lyse the erythrocytes and cells were pelleted by centrifugation. The cells were washed again with sterile PBS and spread on to clean coded slides and stained with freshly prepared ethidium bromide and acridine orange (1:1) stain (Sigma Aldrich Chemical Co. Bangalore, India) and observed under a DM-2500 fluorescent microscope (Leica Microsystems, Wetzlar, Germany).The number of live, and apoptotic cells were counted. A total of 1000 cells were counted for each slide and a total of 5000 cells were counted for each group. The percentage of apoptotic cells was calculated as follows:

Apoptotic index (%) = Number of apoptotic cells scored X 100/Total number of cells counted.

The viable cells were recognized by green fluorescent nuclei with organized structure, whereas the early apoptotic cells showed highly condensed or fragmented yellow chromatin in the nuclei. The cells showing orange chromatin, highly condensed and fragmented nuclei were considered as late apoptotic cells. The apoptotic cells also exhibited membrane blabbing as one of the morphological

features. Only cells with yellow, condensed, or fragmented nuclei were counted as apoptotic cells in a blinded, non-biased manner.

2.10. Biochemical Assays

The alteration in biochemical profile after administration with 200mg/kg b.wt. CGE was assayed by conducting a separate experiment where grouping and other conditions were essentially similar to that described for apoptosis assay. The animals were sacrificed after nine days of drug treatment at an interval of 2, 4, 6, 8, 12 and 24 hours. Both the treated and untreated Dalton's lymphoma cells were aspirated under sterile conditions, washed with ammonium chloride followed by sterile phosphate buffer saline and pelleted. The cell pellets were weighed and 10% homogenate was prepared in cold sterile PBS (pH 7.4) and used for the estimation of various antioxidant and lipid peroxidation.

2.10.1. Estimation of Glutathione

Glutathione contents were estimated as described earlier (Moron *et al.,* 1929). In brief, 1.8 ml of 0.2 M Na_2HPO_4 was mixed with 40 µl of 10 mM DTNB and 160 µl of cell homogenate and allowed to stand for 2 minutes. The absorbance was read against the blank at 412 nm in a UV-VIS Biospectrophotometer (Eppendorf India Limited, Kolkata, India). The blank consisted of distilled water instead of cell homogenate.

2.10.2. Estimation of Glutathione - S – Transferase

Glutathione-s-transferase was determined by the method of Habig *et al.,* (1987). Usually, 0.5 ml of 0.1 M phosphate buffer (pH 6.5), 0.1 ml of 20mM CDNB,

and 8.8 ml distilled water were incubated at 37°C for 10 min followed by the addition of 0.5 ml of 20 mM GSH and 0.1 ml of cell homogenate. The absorbance was read at 340 nm with a UV-VIS spectrophotometer at 1 min intervals for 6 minutes. Distilled water was used as a blank.

2.10.3. Catalase Assay

Catalase was assayed according to technique described by Aebi (1984). The 20 µl of cell homogenates was mixed with 1.98 ml of 50 mM phosphate buffer (pH 7.0) in a 3 ml cuvette. The reaction (maintained at 20ºC) was started by adding 1 ml of 30 mM H_2O_2. The decrease in absorbance was monitored at 240 nm for 60 seconds.

2.10.4. Superoxide Dismutase Assay

The activity of SOD was estimated as described by Fried (1975). 100 µl of cell homogenate, 100 µl of 186 µM phenazenemethosulfate, 300 µl of 3.0 mM nitrobluetetrazolium, and 200 µl of 780 µM NADH were incubated for 90 seconds at 30°C. The reaction was terminated by the addition of 1000 µl of acetic acid and then 4ml n-butanol. The absorbance was read at 560 nm using UV/VIS spectrophotometer. The percent inhibition was calculated by measuring the absorbance of blank without SOD enzyme. The SOD activity was calculated using the formula (Blank-Sample)/Blank X 100.

2.10.5. Lipid Peroxidation Assay

Malondialdehyde (MDA) formed from the breakdown of polyunsaturated fatty acids, serve as a convenient index for determining the extent of peroxidation

reaction of lipids. MDA has been identified as the product of lipid peroxidation (LOO) that reacts with thiobarbituric acid to give a red species absorbing at 535 nm. LOO assay was carried out following the method of Buege and Aust, 1978. One ml of cell homogenate was added to 2 ml of TCA-TBA-HCl reagent and was mixed thoroughly and heated in a boiling water bath for 15 minutes, cooled immediately at room temperature and centrifuged at 1000 rpm for 10 min. The supernatant was collected and its absorbance was read at 535 nm against blank in a UV-VIS spectrophotometer. The blank contained all the reagents except the cell homogenate substituted with distilled water. The MDA concentration of the sample was calculated using the extinction coefficient of $1.56x10^{6}M^{-1}cm^{-1}$.

2.11. Determination of Liver and Kidney function

An experiment was conducted to study the toxic effect of CGE where grouping and other conditions were similar to that of biochemical assay. A 10% homogenate of liver and kidneys was prepared in PBS using a homogenizer (Remi, India). The aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were estimated in the liver, whereas uric acid and creatinine were measured in the kidney homogenates with the help of commercial available Respons kits using a Respons 910 autoanalyzer (Diagnostic Systems GmbH¸ Holzheim, Germany).

3. Statistical Analyses

The statistical analyses were done using Origin Pro 8 SRO v8.0724 (B724), Northampton, MA, USA. The significance for survival analysis was determined by Kaplan Meier test and student's 't' test was applied for biochemical studies followed

by Tukey's post -hoc tests for multiple comparisons, wherever necessary. The Wilcoxson's signed rank test was utilized for micronucleus and apoptosis assays. The results were confirmed by repetition of the experiments. Test of homogeneity was applied to determine any statistical differences between the repeat experiments. Since no significant differences were observed the data of all experiments were combined and expressed as mean ± standard error of the mean (SEM).

4. RESULTS

The results are expressed as the mean ± standard error of the mean (SEM), wherever required in Tables 1-16 and Figures 1-11.

4.1. Acute toxicity

Oral administration of the different extracts of *Colocasia gigantea* showed no signs of toxicity up to 2 g/kg b. wt. The acute toxicity assay after the intraperitoneal mode of administration was carried out by up and down method. Based on the toxicity, chloroform and aqueous extracts showed an LD_{50} of 625mg/kg b. wt. and 710 mg/kg b. wt. respectively, whereas ethanol extract was less toxic as the LD_{50} was823mg/kg b. wt. (Table 4-5).

4.2. Body weight changes

The mice transplanted with DLA cells gained weight continuously due to proliferation of tumor cells until the animal succumbed to death. The tumorized mice did not exhibit signs of tumour regression in the negative control group. The treatment of DLA mice with 50, 75, 100, 125, 150, 175, 200 or 225mg/kg body weight of ethanol extracts of *Colocasia gigantea* exhibited slight elevation in the

body weight (Figure 1). The comparison of *Colocasia gigantea* extract treated groups with negative control revealed a significant reduction in the body weight due to decrease in the cell proliferation (Table 6).

4.3. Anticancer activity

Dalton's lymphoma transplanted mice developed tumour rapidly with no signs of regression and all the untreated tumorized mice died within 18-20 days (Table 7). The AST and MST for this group were found to be 17.33 and 17.5 days, respectively (Table 5; Figure 4).

The treatment of tumorized mice with 50, 75, 100, 125, 150, 175, 200 or 225 mg/kg body weight CGE caused a significant ($p<0.001$) rise in the number of survivors when compared to spontaneous control group (Table 7; Figure 2). A 30% rise in survival was observed in animals treated with 50 mg/kg CGE by 20 days. Time of survival increased with increasing dose up to 175 mg/kg where 20% animals survived up to 48 days and all animals succumbed to death after 54 days (Table 7). A further increase in CGE dose elevated the survival of animals up to 50% until 60 days (Table 7). The AST of 44 days, MST of 60.5 days were reported for 200 mg/kg CGE leading to an IMLS of 211.27% and an IALS of 152.16% , respectively (Table 8; Figure 3).

4.4. Micronucleus Assay

The frequency of micronuclei bearing mononucleate (MNMNC) and binucleate cells (MNBNC) with one and two micronuclei has been represented separately (Table 9, Figure 4(a) & 4 (b)). Treatment of Dalton's lymphoma bearing

mice with CGE or DOX showed a time dependent rise in the frequency of micronuclei ($p<0.001$) in a manner up to 36h post-drug treatment , in both the mononucleate and binucleate cells (Figure 4). The CGE treatment not only induced mononucleated and binucleated cells bearing one micronuclei but also the cells bearing two micronuclei (Figure 4(a) & 4 (b)).

4.5. Apoptosis Assay

The administration of CGE or DOX induced apoptosis in Dalton's lymphoma cells as early as 12 h post drug treatment in a time dependent manner (Figure 5). The number of apoptotic cells in CGE treated DLA cells significantly ($p<0.001$) increased when compared to concurrent control group at all the post CGE treatment times and maximum number of apoptotic cells were reported at 36 h post-treatment (Table 10). This increase in apoptotic index was 14 fold higher for the all the post CGE treatment times (Table 10).

4.6. **Biochemical Assays**

4.6.1. Glutathione content

The treatment of DLA mice with 200mg/kg b. wt. CGE led to a significant decrease in the glutathione contents since 2 h post treatment and it continued t decline up to 24 h post treatment, where the reduction in GSH concentrations was highest (Figure 6). The difference in this alleviation in GSH contents between 8, 12 and 24 h was non-significant (Table 11). The CGE treatment reduced the GSH contents comparable to DOX treatment (Table 11).

4.6.2. Glutathione - S – Transferase (GST)

The GST activity declined significantly ($p<0.001$) in the DLA mice treated with 200 mg/kg b. wt. CGE (Table 12). The GST activity showed a time dependent decline and the maximum decline was found at 24 h post CGE treatment (Figure 7). The DOX treatment also showed a similar decline in GST activity (Figure 7).

4.6.3. Catalase (CAT) activity

Administration of CGE and DOX led to a gradual and time dependent decline in the catalase activity until 24 h post treatment (Figure 8), where it was 1.4 fold lower than the SPS treatment (Table 13). The decline in the GST activity was significant ($p<0.001$) when compared to negative SPS treatment (Table 13).

4.6.4. Superoxide dismutase (SOD) activity

The SOD activity decreased in a time dependent manner in the DLA mice treated with CGE or DOX treatment until 6 h post treatment where a greatest reduction in SOD activity was observed after CGE administration (Figure 9). This decline was approximately 2.3 and 3 fold for CGE and DOX treatment, respectively when compared to negative SPS control at 6 h post treatment (Table 14). The SOD activity increased with time after 6 h but did not reach to negative control level even at 24 h post treatment where it was 2 fold lower (Table 14).

4.6.5. Lipid peroxidation (LOO)

Treatment of DLA mice with 200 mg/kg b. wt. CGE led to 3 fold elevation in the lipid peroxidation as early as 1 h post –treatment (Table 15) when compared to

SPS group. Increase in assay time resulted in a further rise in LOO and 3.6 fold elevation was recorded at 6 h post treatment in the CGE group (Table 15). The LOO increased in a time dependent manner up to 6 h post treatment in both CGE and DOX treated group and started to increase gradually until 24 h post treatment (Figure 10) where LOO was still higher than the SPS treatment (Table 15).

4.6.6. Liver and Kidney function tests

The intraperitoneal administration of CGE (200 mg/kg b. wt.) for consecutive 9 days did not significantly alter aspartate aminotransferase (AST), and alanine aminotransferase (ALT) in the liver and creatinine and uric acid in the kidney of mice (Table 16 and Figure 11). Therefore, the treatment of 200 mg/kg body weight of CGE did not cause undesirable effect on the liver.

DISCUSSION

Chemotherapy is one of the most preferred modality to treat cancer, especially when patients have metastasis. Despite increased survival and cure rates, chemotherapy increases toxicity in the normal tissues and also rapidly dividing tissues leading to morbidity and mortality (Coleman and Mitchell, 1999; Bhandare and Mendenhall, 2012; Lawrence *et al.,* 2014).The definite therapy to reduce the toxic effects of chemotherapy is not yet available and efforts to reduce adverse toxic side effects without compromising their efficacy to cure tumors shall continue. Herbal medicines have been practiced in the world since the advent of human history and their scientific evaluation may help to develop new pharmacophores that can be used as modern therapeutic agents to cure cancer. The use of herbal medicines as

adjuvant may be reduce the toxic side effects of chemotherapy and increase its efficacy on neoplastic cells at the same time protecting the normal tissue from chemotherapy-induced toxic side effects. The herbal drug may enhance the immune surveillance of normal tissues, which are affected adversely during neoplastic transformation. The inclusion of herbal medicine in chemotherapy may improve the therapeutic index by killing neoplastic cells and reducing the toxicity to normal tissues (Tannock, 1996). The natural products may play an important role by killing neoplastic cells and not allowing the normal cells to transform into the malignant phenotype. The advantage of natural products is that they are natural in origin and hence most biocompatible with minimum side effects in comparison to chemical synthetic products (Jagetia, 2007). Therefore, the present study was undertaken to evaluate the ability of *Colocasia gigantea* to kill the Dalton's lymphoma cells transplanted in mice.

Different extracts of *Colocasia gigantea* administered with a single oral dose showed no signs of any toxicity up to 2 g/kg b.wt. in Swiss albino mice hence it can be considered safe orally. However, the intraperitoneal mode of administration revealed significant toxicity, where the chloroform, ethanol and aqueous extracts showed LD_{50} of 625 mg/kg b. wt., 823 mg/kg b. wt. and 710 mg/kg b.wt., respectively, after intraperitoneal administration. In an earlier study the LD_{50} for interaperitoneal administration was found to be lower than oral admiration for leaf extract of *Blighia unijugata* (Frédéric *et al.,* 2013).

Assessment of antitumour activity on Dalton's lymphoma transplanted intraperitoneally indicated that DLA cells grew rapidly in the untreated mice and all

the tumorized mice died within 18-20 days with an average survival time (AST) and median survival time (MST) of 17.33 and 17.5 days respectively. Treatment of DLA mice with different doses of CGE led to a rise in the survival of mice in a dose dependent manner and a maximum number of survivors were observed at a dose of 200 mg/kg b. wt. with a 50% tumor free survivors beyond 120 days. The increase in tumor free survivors have been reported for the stem bark extract of *Alstonia scholaris, Aphnamixis polystachya, Ervatamia heyncana, Hygrophila spinosa, Podyphyllum hexandrum, Rubia cordifolia, Tinospora cordifolia* and *Tylophora indica* earlier (Chitnis *et al.,* 1971; 1972; 1979; Adwankar *et al.,* 1980; Mazumdar *et al*., 1997; Jagetia *et al.,* 1997; 2006; Goel *et al.,* 1998; Jagetia, 2008; Jagetia and Venkatesha, 2012).

The infliction of DNA damage is one of the important events to kill tumor cells and many chemotherapeutic agent induce DNA damage to kill neoplastic cells (Cheung-Ong *et al.,* 2013). The ability of ethanol extract of *Colocasia gigantea* to trigger the DNA damage was tested in the tumorized mice and it was found that CGE induced DNA damage as evidenced by the increase in the formation of micronuclei in mononucleated as well as binucleated cells effectively. Treatment of Dalton's lymphoma bearing mice with CGE showed a time dependent elevation in the frequency of micronuclei up to 36 h post treatment.. A similar effect has been observed earlier (Adiga and Jagetia, 1999; Jagetia and Venkatesh, 2015; Jagetia and Venkatesha, 2016).The CGE induced not only one micronuclei but also cells with two micronuclei indicating that it induced complex DNA damage in the form of multiply damaged sites that would have repressed the DNA damage repair leading to

higher cell death. A number of studies have indicated that the cells expressing micronuclei are dying cells and correlation between cell killing and micronuclei has been reported (Adiga and Jagetia, 1999; Jagetia *et al.,* 2007; Jagetia and Venkatesh, 2015; Jagetia and Venkatesha, 2016). The micronuclei assay provide an indirect measure of DNA damage since the micronuclei arise due to defective cell division, mis-segregation of chromosomes, DNA exchanges and faulty or suppressed DNA repair leading to cell death (Jagetia *et al.,* 2007; Sage and Harrison, 2011; Jagetia and Rao, 2011, 2015; Yates and Campbell, 2012; Zhang *et al.,* 2015; Jagetia and Venkatesh, 2015; Jagetia and Venkatesha, 2016;). The formation of DNA DSBs and micronuclei is often the consequence of simultaneous excision repair of damages, wrong base incorporation and failure of the appropriate gap-filling event leads to DSB (Dianov *et al.,* 1991). This may happen only if the level of DSBs exceeds the repair capacity of dividing cells, which is mainly due to either the misrepair of DSBs by the dysfunctional homologous recombination (O'Donovan and Livingston, 2010).

The apoptosis induction is a silent form of cell death and many chemotherapeutic agents induce apoptosis to shrink the tumor (Kaufmann and Earnshaw, 2000; Seitz *et al.,* 2009). One of the important cause of cell death by CGE seems to be induction of apoptosis. Treatment of DLA mice with CGE triggered apoptosis in a time dependent manner leading to increased tumor free survivors in the present study. The infliction of DNA damage in the cells by CGE may have triggered a cascade of biochemical and molecular events inducing apoptosis, which was characterized by chromosome condensation, DNA fragmentation, membrane blebbing and formation of apoptotic bodies and cell death (Cotter, 2009; Nikoletopoulou *et al.,* 2013).

Chapter 5

The cancer cells are always at higher oxidative stress and a further increase in oxidative stress will stimulate cells to undergo DNA damage and thus it is able to kill tumor cells effectively. The chemotherapeutic agents are known to kill tumor cells by modulating oxidative stress (Conklin, 2004; Gorrini *et al.,* 2013). The treatment of tumor bearing mice has reduced the glutathione concentration as increased GSH is involved in resistance to apoptosis and also to chemotherapy, whereas reduced glutathione sensitizes cancer cells to death (Circu and Aw, 2012; Franco and Cidlowski, 2012; Traverso *et al.,* 2013; Rocha *et al.,* 2014; Ramsay and Dilda, 2014). Gultahione is an important biomolecules synthesized by cells and it plays crucial role in the detoxification, cell differentiation, proliferation and apoptosis however, reduced GSH levels cause oxidative stress (Meister and Anderson, 1983; Ganesaratnam *et al.,* 2004; Lushchak, 2012; Traverso *et al.,* 2013; Schumacker, 2015). The cell killing effect of CGE may be due to its ability to reduce glutathione concentration in the tumor cells. Similarly, treatment of Dalton's lymphoma with CGE had a negative effect on the activities of GST, catalase and SOD, which are also involved in resistance to chemotherapy (Kodydková *et al.,* 2014; Zeng *et al.,* 2014; Che *et al.,* 2016). This depletion in their activities may have made tumors cells susceptible to the cytotoxic effect of CGE causing increased tumor free survival in the present study.

The lipid peroxidation is involved in increased oxidative stress and cell death when chemotherapeutic agents come into the contact of cancer cells. Since lipid are integral part of cell membrane and their peroxidation damages the cell membrane kills the cells effectively (Barrera, 2012; Gaschler and Stockwell, 2017). The

increase of lipid peroxidation in DLA cells by CGE may have killed the tumor cells by damaging their membrane and inducing damage ot proteins and nucleic acids (Gaschler and Stockwell, 2017).

The mechanism of tumor cell kill by CGE is not well understood. However, employment of multiple putative pathways to kill tumor cells seems to be operational in the present study. The increase in lipid peroxidation may have changed cell membranes and also the important macromoluelcues like DNA and proteins kill the tumor cells and increasing the tumor free survivors in the present study. The CGE has actually increased the DNA damage in both mono and binucleated tumor cells and also induced apoptosis which supports the above contention. The reduction in GSH, GST, catalase and SOD by CGE would have increased the oxidative stress in the DLA cells bringing effective cell killing in the present study. At molecular levels negative modulation of Nrf2, COX-II and NF-κB by CGE may have favoured the tumor cell killing as the activation of these genes is involved in failure of tumor therapy (Sobolewski *et al.,* 2010; Lu and Stark, 2015; Choi and Kwak, 2016). Since CGE induced apoptosis it may have stimulated apoptotic cascade by upregulating p53, caspases, Bax and other proteins in the present study. Some other unknown mechanisms may also have contributed to the cytotoxic effect of CGE.

CONCLUSIONS

The CGE killed tumor cells and increased the tumor free survival, which may be due to its ability to induce DNA damage and it increased micronuclei and apoptosis. The apoptosis may have been triggered by the activation of p53, Bax and p21 and Caspases 3. It may have also suppressed the transcriptional activation of

Nrf2, COX-II and NF-κB. The elevation in lipid peroxidation and depletion in GSH, GST, catalase and SOD may have played a major role in inducing DNA damage and stimulating apoptotic pathway that finally killed the DLA cells and increased the tumor free survivors in CGE treated mice beyond 120 days.

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Table 1: The survival of mice administered intraperitoneally with different doses of chloroform extract of *Colocasia gigantea* rhizomes.

Dose (g/kg b. wt.)	Mortality (%) 0n different days														Total	Remarks
	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
3	100	-	-	-	-	-	-	-	-	-	-	-	-	-	0	Aggressive, dull and all died within first day.
2.5	-	-	30	-	-	20	-	-	-	20	-	-	-	-	30	Aggressive, dull and 70% died before 14 days.
2	-	30	-	-	-	-	10	-	-	-	20	-	-	-	40	Aggressive, dull and 60% died before 14 days.
1.5	-	-	20	-	-	-	20	-	-	10	-	-	-	-	50	Dull, lethargic and 50 % died before 14 days.
1	-	20	-	-	-	10	-	-	-	10	-	-	-	-	60	Dull, lethargic and 60 % died before 14 days.
0.5	-	-	-	10	-	-	-	-	-	-	-	10	-	-	80	Dull and 20 % died before 14 days.
0.2	-	-	-	10	-	-	-	-	-	-	-	-	-	-	90	Active and 10% died before 7 days.
0.15	-	-	-	-	-	-	10	-	-	-	-	-	-	-	90	Active and 10% died on 7 days.
0.1	-	-	-	-	-	-	-	-	-	-	10	-	-	-	90	Active and 10% died before 14 days.
0.05	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100	Active and all survived.

N=10 for each dose

Table1 2: The survival of mice administered intraperitoneally with different doses of ethanol extract of *Colocasia gigantea* rhizomes.

Dose (g/kg b. wt.)	Mortality (%) on different days														Total	Remarks
	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
3	10	-	-	-	-	-	-	-	-	-	-	-	-	-	0	Aggressive, Dull and died within first day.
2.5	-	30	-	20	-	-	20	-	-	-	20	-	-	-	30	Aggressive, dull and 70% died before 14 days.
2	-	-	20	-	-	-	-	-	20	10	-	-	-	-	50	Aggressive, dull and 50% died before 14 days.
1	-	-	10	-	-	-	-	-	20	-	-	-	-	-	60	Dull, lethargic and died before 14 days.
0.5	-	-	-	-	-	-	-	-	-	10	-	-	-	-	80	Dull, lethargic and 40% died before 14 days.
0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	90	Dull and died before 7 days.
0.15	-	-	-	-	10	-	-	10	-	-	-	-	-	-	90	Dull and died before 10 days.
0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	90	Active and 10% died before 14 days.
0.05	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100	Active and all survived.

N=10for each dose

Table 3: The survival of mice administered intraperitoneally with different doses of aqueous extract of *Colocasia gigantea* rhizomes.

Dose (g/kg b. wt.)	Mortality (%) 0n different days														Total	Remarks
	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
3	100	-	-	-	-	-	-	-	-	-	-	-	-	-	0	Aggressive, dull and all died within 3 hrs.
2.5	100	-	-	-	-	-	-	-	-	-	-	-	-	-	0	Aggressive, dull and all died within first day.
2	20	-	-	-	30	-	-	-	10	-	-	-	10	-	30	Aggressive, dull and 70% died before 14 days.
1.5	-	20	-	-	-	-	10	-	-	-	-	20	-	-	50	Dull, lethargic and 50 % died before 14 days.
1	-	10	-	-	-	-	20	-	-	10	-	-	-	-	60	Dull, lethargic and 60 % died before 14 days.
0.5	-	-	-	-	20	-	-	-	-	-	-	-	-	-	80	Dull, and 20 % died before 7 days.
0.2	-	-	-	10	-	-	-	-	-	-	-	-	-	-	90	Active and 10% died before 7 days.
0.15	-	-	-	-	-	-	10	-	-	-	-	-	-	-	90	Active and 10% died on 7 days.
0.1	-	-	-	-	-	-	-	-	-	10	-	-	-	-	90	Active and 10% died before 14 days.
0.05	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100	Active and all survived.

N=10 for each dose

Table 4: Acute toxicity of different solvent extracts of *Colocasia gigantea* administered orally in Albino mice.

Extract/ Group	Sex	Dose (g/kg.bwt.)	Body weight (g)			Survival
			Before fasting	After fasting	Loss (18 h)	
Control (SPS)	M	0	30	27	3	> 14 days
			32	29.8	2.2	> 14 days
			28.2	25	3.2	> 14 days
	F		30	28.9	1.1	> 14 days
			25.8	23.2	2.6	> 14 days
			27	24	3	> 14 days
Chloroform	M	2	29.4	27.5	1.9	> 14 days
			33	30	3	> 14 days
			29.3	27.4	1.9	> 14 days
			31	29.6	1.4	> 14 days
			29.6	27.5	2.1	> 14 days
	F		27	24	3	> 14 days
			29.6	26.3	1.3	> 14 days
			29	27	2	> 14 days
			26.2	25	1.9	> 14 days
			27.6	25.7	1.8	> 14 days
Ethanol	M	2	34.2	31.3	2.9	> 14 days
			32	30.6	1.4	> 14 days
			29.7	28.6	1.1	> 14 days
			27.5	25.8	1.7	> 14 days
			28.7	27.5	1.2	> 14 days
	F		33.1	29	4.1	> 14 days
			30.7	28.7	2	> 14 days
			32.4	30.4	2	> 14 days
			35.4	31	4.4	> 14 days
			32.8	30.8	2	> 14 days
Aqueous	M	2	35.3	33.2	2.1	> 14 days
			33.6	30.6	3	> 14 days
			34.5	32	2.5	> 14 days
			29.7	28.3	1.4	> 14 days
			28.8	26.4	2.4	> 14 days
	F		30.5	28.7	1.8	> 14 days
			31.6	28.8	2.8	> 14 days
			33.6	31.7	1.9	> 14 days
			28.7	28.9	1.8	> 14 days
			29.6	27.7	1.9	> 14 days

N=10 for each dose

Table 5: Determination of acute toxicity in Swiss albino mice administered various doses of different extracts of *Colocasia gigantea* intraperitoneally.

Extract type	Dose (mg/kg b. wt.)	Survival (%)	LD_{50} (mg/kg)
Chloroform	50	100	**625**
	100	90	
	150	90	
	200	90	
	500	80	
	1000	60	
	1500	50	
	2000	40	
	2500	30	
	3000	0	
Ethanol	50	100	**823**
	100	90	
	150	90	
	200	90	
	500	80	
	1000	60	
	2000	50	
	2500	30	
	3000	0	
Aqueous	50	100	**710**
	100	90	
	150	90	
	200	90	
	500	80	
	1000	60	
	1500	50	
	2000	30	
	2500	0	
	3000	0	

The LD_{50} is determined using Probit analysis.

N=10 for each dose

Table 6: Change in body weight of Dalton's lymphoma bearing Swiss albino mice.

Dose (mg/kg.b.wt)	Body weight (g)±SEM												
	Post tumour transplanted time (day)												
	0	3	6	9	12	15	18	21	24	28	30	33	36
0	25.97±0.62	26.34±0.67	27.4±0.59	28.55±0.59	29.36±0.56	30.39±0.61	30.26±0.96						
50	25.97±0.62	26.34±0.67	27.4±0.59	28.55±0.59	29.36±0.56	30.39±0.61	31.06±0.58	31.42±0.59	31±0.62				
75	29.66±0.95	30.04±0.98	31.2±0.94	32.12±0.92	33.21±0.95	33.84±0.90	34.78±0.82	35.25±0.91	34.37±0.55	34.5±0.51	29.66±0.95	30.04±0.98	31.2±0.94
100	29.82±0.67	30.46±0.65	31.2±0.65	33.01±0.64	32.7±0.74	33.4±0.73	33.6±0.71	33.52±0.64	33.97±0.64	34.77±0.70	34.2±0.51	35.15±0.31	35.7±0±.67
125	29.78±0.39	30.37±0.36	31.3±0.41	32.21±0.38	33.53±0.46	34.71±0.59	35.86±0.76	37.01±0.89	36.42±0.82	37.52±0.90	37.2±1.09	29.78±0.39	30.37±0.36
150	29.29±0.31	30.03±0.30	31.16±0.32	32.42±0.45	32.42±0.48	34.25±0.48	34.72±0.39	35.67±0.40	36.62±0.39	38.1±0.47	39.36±0.51	40.36±0.61	40.7±0.51
175	28.85±0.33	29.11±0.33	30.11±0.44	31.3±0.37	31.94±0.41	33±0.45	33.51±0.36	34.37±0.38	34.98±0.24	35.82±0.23	36.75±0.32	37.9±0.42	38.91±0.51
200	28.73±0.26	30±0.18	31.01±0.27	32.18±0.28	33.82±0.36	35.5±0.52	37.5±0.52	39.2±0.59	41.2±0.29	42.2±0.25	44.05±0.02	45±0.26	45.6±0.18
225	29.29±0.31	29.58±0.30	30.67±0.18	31.87±0.30	32.87±0.37	33.63±0.35	34.5±0.35	35.48±0.26	36.66±0.31	37.22±0.27	38.14±0.32	39.02±0.43	39.67±0.60

N=10 for each dose

Table 7: Effect of ethanol extract of *Colocasia gigantea* on Survival of Dalton's lymphomas ascites bearing mice treated with various doses administered intraperitoneally, N=10.

Post tumor transplant time (day)	SPS (Control)	Survivality (%)							
		Ethanol Extract							
		Dose (mg/kg.b.wt)							
		50	**75**	**100**	**125**	**150**	**175**	**200**	**225**
0	100	100	100	100	100	100	100	100	100
18	30	90	90	60	90	50	80	100	90
20	0	90	90	60	90	50	80	100	90
22	0	70	80	40	80	40	80	90	60
24	0	10	40	40	40	40	70	90	60
26	0	0	40	40	40	40	70	90	60
28	0	0	20	20	40	30	70	80	50
30	0	0	0	20	20	30	70	80	50
32	0	0	0	20	2 0	30	70	80	50
38	0	0	0	0	10	2 0	70	70	40
40	0	0	0	0	0	0	40	70	20
44	0	0	0	0	0	0	40	60	20
46	0	0	0	0	0	0	20	60	10
48	0	0	0	0	0	0	20	60	0
54	0	0	0	0	0	0	0	60	0
60	0	0	0	0	0	0	0	50	0
120	0	0	0	0	0	0	0	50	0

Table 8: Effect of ethanol extract of *Colocasia gigantea* on Dalton's lymphoma ascites bearing mice and the tumor response assessment based on median survival time (MST) and average survival time (AST).Increase in mean life span (% IMLS) and increase in average life span (% IALS). The results were expressed as percent (%) ± SEM, N=10

Treatment	Dose (mg/kg.b.wt.)	MST	AST	IMLS(%)	IALS(%)
Control (SPS)	0	17.5±0.33	17.33±0.32	0.05±0.00	0.05±0.00
Ethanol	50	20.25±1.75*	20.5±0.28*	30.3±1.24#	31.63±0.65#
	75	22.45±1.13*	23.5±1.21*	40.42±1.18#	41.88±1.68#
	100	24.55±1.24*	24.75±1.35*	46.48±2.11#	49.53±1.055#
	125	26.25±1.25*	27.4±0.65*	64.67±1.46#	65.76±2.24#
	150	27.5±1.21*	28.5±1.14*	66.47±1.24#	68.94±1.44#
	175	35.5±1.15*	36.75±0.25*	131.33±1.46#	129.12±1.27#
	200	60.55±1.15*	44±0.12*	211.27±1.69#	152.16±1.29#
	225	45.5±1.18*	58.22±0.65*	276.79±1.58#	260.59±1.09#

**p<0.001, #p<0.0001 when treatment are compared to control group.*

Table 9: Frequency of micronuclei in Dalton's lymphoma ascites bearing mice treated with 200mg/kg b.wt. ethanol extract of *Colocasia gigantea* (CGE) or 0.5mg/kg b.wt. doxorubucin (DOX) at different post treatment times.

Cell type	Post treatment time (h)	Micronucleated cells/1000±SEM								
		SPS			CGE 200			DOX 0.5		
		One MN	Two MN	Total	One MN	Two MN	Total	One MN	Two MN	Total
Mononucleated cell	0	1.8±0.37	0.1±0.37	2.6±0.51	2.2±0.37	1.2±0.37	1.7±0.34	3.2±0.20	1.4±0.24	2.3±0.12
	12	4.2±0.37	1.4±0.51	5.6±1.51	17±1.73	6.8±o.58	22.8±0.37	43±1.45	8.4±0.81	45.6±0,75
	24	5.4±0.51	1.6±0.40	7±0.70	52±2.0	12.2±0.37	58.2±1.02	73±2.42	21.4±0.81	88.8±1.24
	36	6.4±0.24	1.8±0.49	8.2±0.37	71±2.0	22.8±0.58	91±0.95	101±2.31	32.6±0.81	119.8±0.91
		One BN	**Two BN**	**Total**	**One BN**	**Two BN**	**Total**	**One BN**	**Two BN**	**Total**
Binucleated cell	0	4.2±0.37	0.8±0.37	5±0.89	51.6±0.51*	8.2±0.37*	59.8±0.86	60.2±0.37*	10.2±0.37*	71.2±0.66
	12	5.2±0.37	1.2±0.58	6.4±0.92	81.2±0.37*	11.6±0.4*	92.8±0.73	94±0.44*	13.8±0.37*	107.8±0.8
	24	5.6±0.51	1.4±0.51	7±1.00	92.2±0.37*	13.2±0.37*	105.4±0.75	108.6±0.51*	17.8±0.37*	126.4±0.87
	36	5.8±0.58	1.8±0.49	7.6±1.08	90.8±0.37*	15.4±0.24*	106.2±0.58	102±0.54*	20.6±0.51*	122.6±1.03

**p<0.001when treatment are compared with concurrent control (SPS) group.*

No symbol= no significance.

N=5 for each time

Table 10: Apoptotic index in Dalton's lymphoma ascites bearing mice after treatment with 200 mg/kg b.wt. ethanol extract of *Colocasia gigantea* (CGE) or 0.5mg/kg b.wt. doxorubicin (DOX) at different post treatment times.

Post treatment time (h)	Apoptosis (% ± SEM)		
	SPS	CGE 200	DOX 0.5
0	0.48±0.02	0.68±0.03*	0.98±0.07#
12	0.74±0.05	10.96±0.19#	14.12±0.36#
24	0.92±0.08	13.14±0.67#	17.48±0.31#
36	1.02±0.07	14.68±0.57#	18.8±0.24#

**p<0.05, #p<0.001 when treatment are compared with concurrent control (SPS) group.*
No symbol= no significance.

N=5 for each time

Table 11: The effect of 200 mg/kg b.wt. ethanol extract of *Colocasia gigantea* (CGE) or Doxorubicin (DOX) on the glutathione contents in mice bearing Dalton's lymphoma ascites at different post treatment times.

Post treatment time (h)	Glutathione (GSH) (µM/mg protein), Mean ± SEM		
	SPS	CGE	DOX
0	10.63±0.07	10.02±0.07*	8.43±0.04*
2	10.61±0.03	9.33±018*	7.81±0.03*
4	10.63±0.07	9.29±0.31*	7.97±0.14*
6	10.63±0.07	9.15±0.23*	8.17±0.07*
8	10.61±0.03	8.82±0.19*	8.30±0.19*
12	10.63±0.07	8.58±0.2*	8.32±0.36*
24	10.63±0.07	8.26±0.11*	8.38±0.05*

**p<0.001when treatment are compared with concurrent control (SPS) group.*
No symbol= no significance.
N=10 for each time

Table 12: The effect of 200 mg/kg b.wt. ethanol extract of *Colocasia gigantea* (CGE) or doxorubicin (DOX) on the glutathione-s-transferase activity in mice bearing Dalton's lymphoma ascites at different post treatment times.

Post treatment time (h)	Glutathione-S-Transferase (U/mg protein), Mean ± SEM		
	SPS	CGE	DOX
0	0.088±0.004	0.065±0.001*	0.06±0.005*
2	0.083±0.005	0.065±0.003*	0.059±0.002*
4	0.084±0.004	0.057±0.001*	0.054±0.006*
6	0.09±0.009	0.051±0.003*	0.048±0.007*
8	0.086±0.004	0.055±0.005*	0.0468±0.005*
12	0.088±0.004	0.056±0.001*	0.0458±0.001*
24	0.083±0.005	0.054±0.002*	0.047±0.004*

**p<0.001 when treatment are compared with concurrent control (SPS) group.*
No symbol= no significance.
N=10 for each time

Table 13: The effect of 200 mg/kg b.wt. ethanol extract of *Colocasia gigantea* (CGE) or doxorubicin (DOX) on the catalase activity in mice bearing Dalton's lymphoma ascites at different post treatment times.

Post treatment (h)	Catalase (CAT) (U/mg protein), Mean ± SEM		
	SPS	CGE	DOX
0	33.08 ± 0.47	31.58 ± 0.66	28.59 ± 0.74*
2	33.07 ± 0.98	30.51 ± 0.47	26.24 ± 0.53*
4	33.51 ± 0.53	29.23 ± 0.74*	23.68 ± 0.66*
6	33.50 ± 0.68	25.38 ± 0.74*	22.60 ± 0.31*
8	33.93 ± 0.57	25.21± 0.67*	21.47 ± 0.31*
12	33.93 ± 0.67	24.96 ± 0.67*	20.97± 0.27*
24	33.07 ± 0.98	24.32 ± 0.31*	20.32± 0.57*

**p<0.001 when treatment are compared with concurrent control (SPS) group.*
No symbol= no significance.
N=10 for each time

Table 14: The effect of 200 mg/kg b.wt. ethanol extract of *Colocasia gigantea* (CGE) or doxorubicin (DOX) on the superoxide dismutase activity in mice bearing Dalton's lymphoma ascites at different post treatment times.

Post treatment time (h)	Superoxide dismutase (SOD) (U/mg protein), Mean ± SEM		
	SPS	CGE	DOX
0	1.93 ± 0.05	1.74 ± 0.028 *	1.62 ± 0.06*
2	1.905 ± 0.03	1.53 ± 0.02*	1.47 ± 0.01*
4	1.916 ± 0.04	1.03 ± 0.06*	0.89 ± 0.04*
6	1.90 ± 0.069	0.82 ± 0.09*	0.63 ± 0.01*
12	1.93 ± 0.07	0.95 ± 0.05*	0.61 ± 0.03*
24	1.90 ± 0.03	0.93 ± 0.01*	0.58 ± 0.02*

**p<0.001when treatment are compared with concurrent control (SPS) group.*
No symbol= no significance.
N=10 for each time

Table 15: Alterations in the Lipid peroxidation in mice bearing Dalton's lymphoma treated with *Colocasia gigantea* extract (CGE) and Doxorubicin (DOX).

Post treatment time (h)	Lipid Peroxidation (nmol/mg Protein), Mean±SEM		
	SPS	CGE	DOX
0	1.43 ± 0.012	3.17 ± 0.005*	3.57 ± 0.017*
2	1.49 ± 0.01*	4.89 ± 0.02*	8.68 ± 0.015*
4	1.53 ± 0.03*	4.98 ± 0.01*	9.80 ± 0.028*
6	1.79 ± 0.02*	6.39 ± 0.02*	10.79 ± 0.03*
8	1.77±0.03*	6.27±0.05*	10.53±0.03*
12	1.82 ± 0.01*	6.03 ± 0.01*	10.11 ± 0.02*
24	1.99 ± 0.02*	4.68 ± 0.05*	09.94 ± 0.02*

**p<0.0001when treatment are compared with concurrent control (SPS) group.*
No symbol= no significance.
N=10 for each time

Table 16: Effect of *Colocasia gigantea* extract (CGE)and Doxorubicin (DOX) on the liver and kidney function of Dalton's lymphoma bearing mice.

Treatment	Dose (mg/kg.b.wt.)	Post treatment time (h)	Liver function test		Kidney function test	
			AST (U/ml)	ALT (U/ml)	Creatinine (mg%)	Uric acid (mg/dl)
Control (SPS)	0	0	29.11±1.42	18.36±0.72	0.75±0.02	2.74±0.23
		12	29.28±0.94	19.46±1.32	0.58±0.04	2.24±0.35
		24	29.12±2.32	22.24±0.72	0.51±0.08	2.31±0.07
CGE	200	0	33.88±0.35	23.93±0.34	0.75±0.02	3.02±0.02
		12	37.58±0.23	21.03±2.65	0.60±0.01	2.65±0.17
		24	38.16±0.12*	23.44±0.24	0.85±0.06	2.81±0.11
DOX	0.5	0	31.49±0.23	21.88±1.32	0.79±0.06	3.65±0.09
		12	30.50±0.47	24.65±0.25	0.86±0.03*	3.76±0.13*
		24	35.36±0.35*	23.81±0.36	0.87±0.05*	3.81±0.17*

**p<0.05 when treatment group are compared with control group.*
No symbol= no significance.
The results are the Mean ± SEM.
N=10 for each time

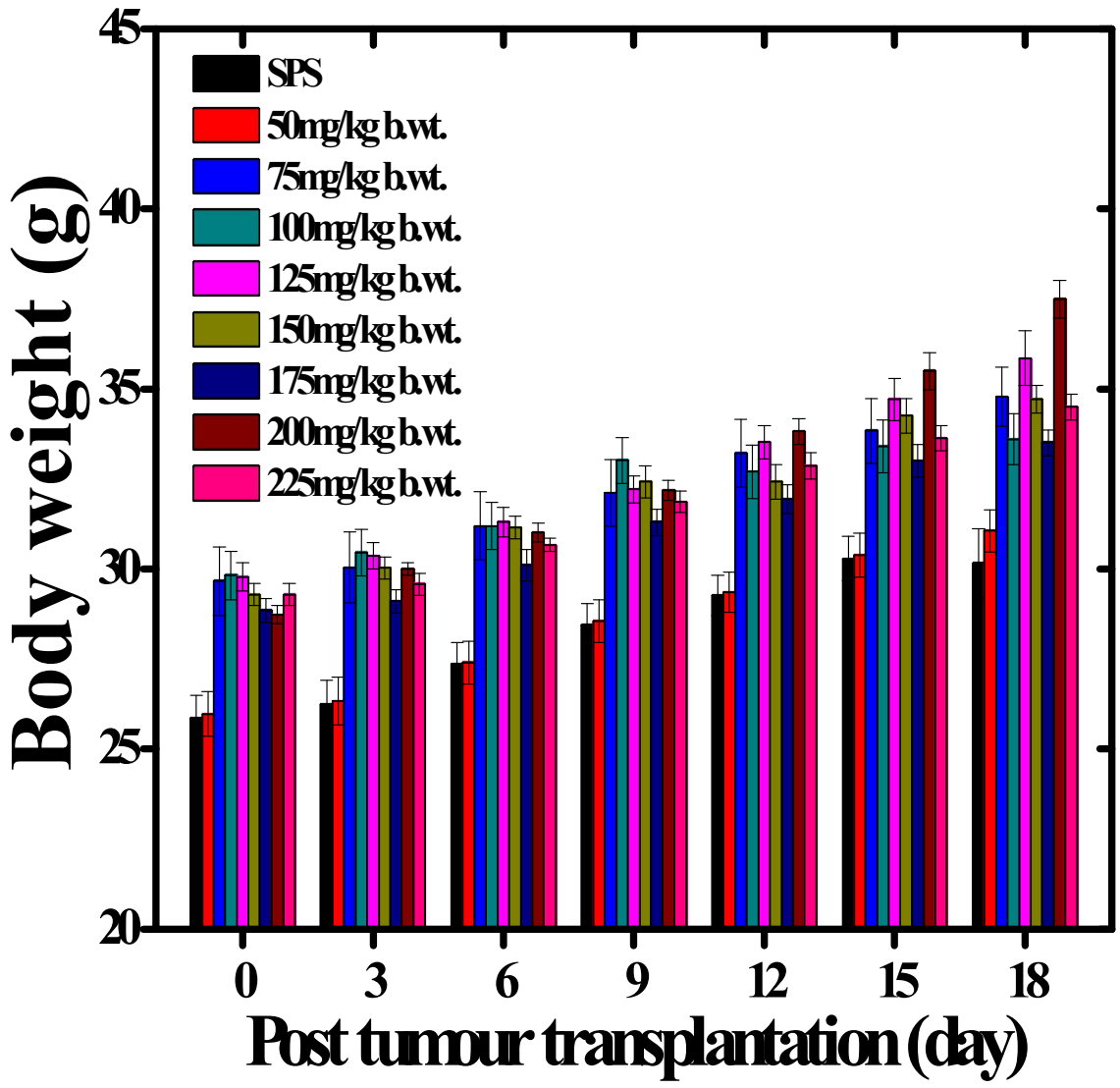


Figure 1: Body weight change of Dalton's lymphoma ascites bearing Swiss Albino mice by treating with ethanol extract of *Colocasia gigantea.* Black bar: SPS; Red bar: 50mg/kg b.wt.; Blue bar: 75mg/kg b.wt.; Sea-green bar: 100mg/kg b.wt.; Purple bar: 125mg/kg b.wt.; Olive-green bar: 150mg/kg b.wt.; Navy-blue bar: 175mg/kg b.wt.; Maroon bar: 200mg/kg b.wt.; Pink bar: 225mg/kg b.wt. N=10.

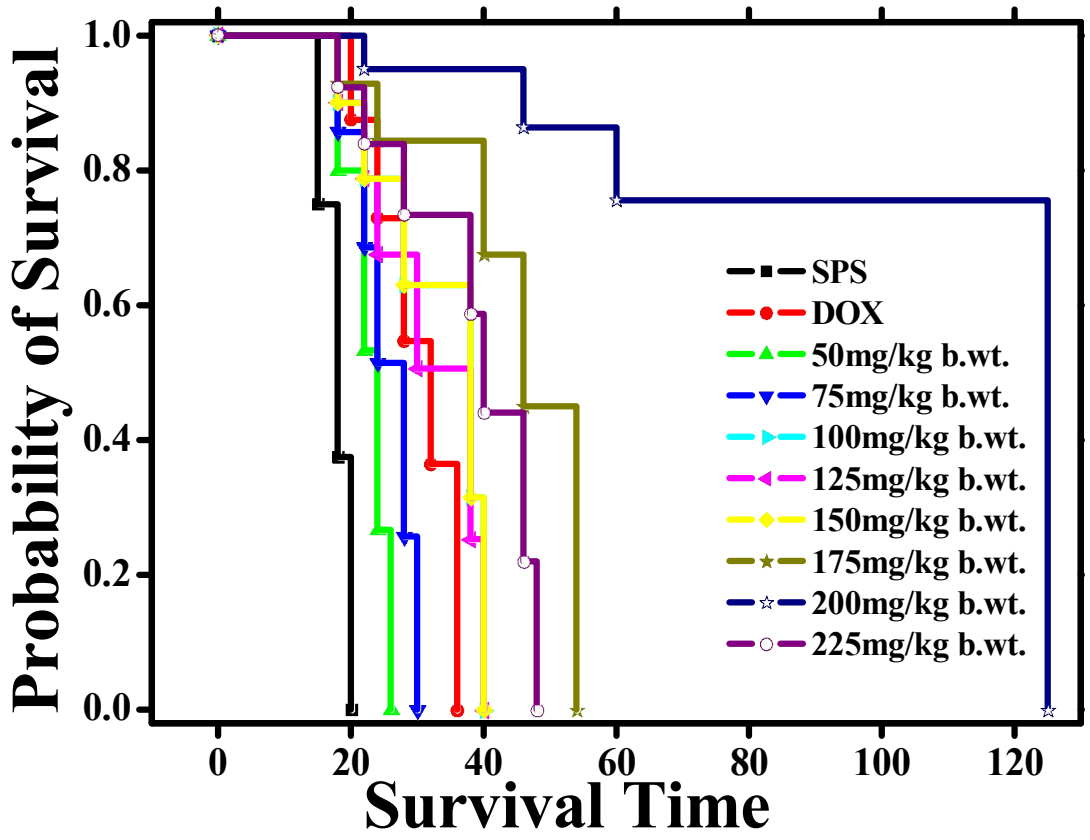


Figure 2: The survival of Dalton's lymphoma ascites bearing mice treated for 9 days consecutively with SPS or doxorubicin and different doses of ethanolic extract of *Colocasia gigantea*. Closed square: SPS; Closed circle: Doxorubicin (DOX); Closed triangle up: 50mg/kg b.wt.; Closed triangle down: 75 mg/kg b.wt.; Closed triangle right: 100 mg/kg b.wt.; Closed triangle left: 125 mg/kg b.wt.; Closed diamond: 150 mg/kg b.wt.; Closed star: 175 mg/kg b.wt.; Opened star: 200 mg/kg b.wt.; Opened circle: 225 mg/kg b.wt. N=10

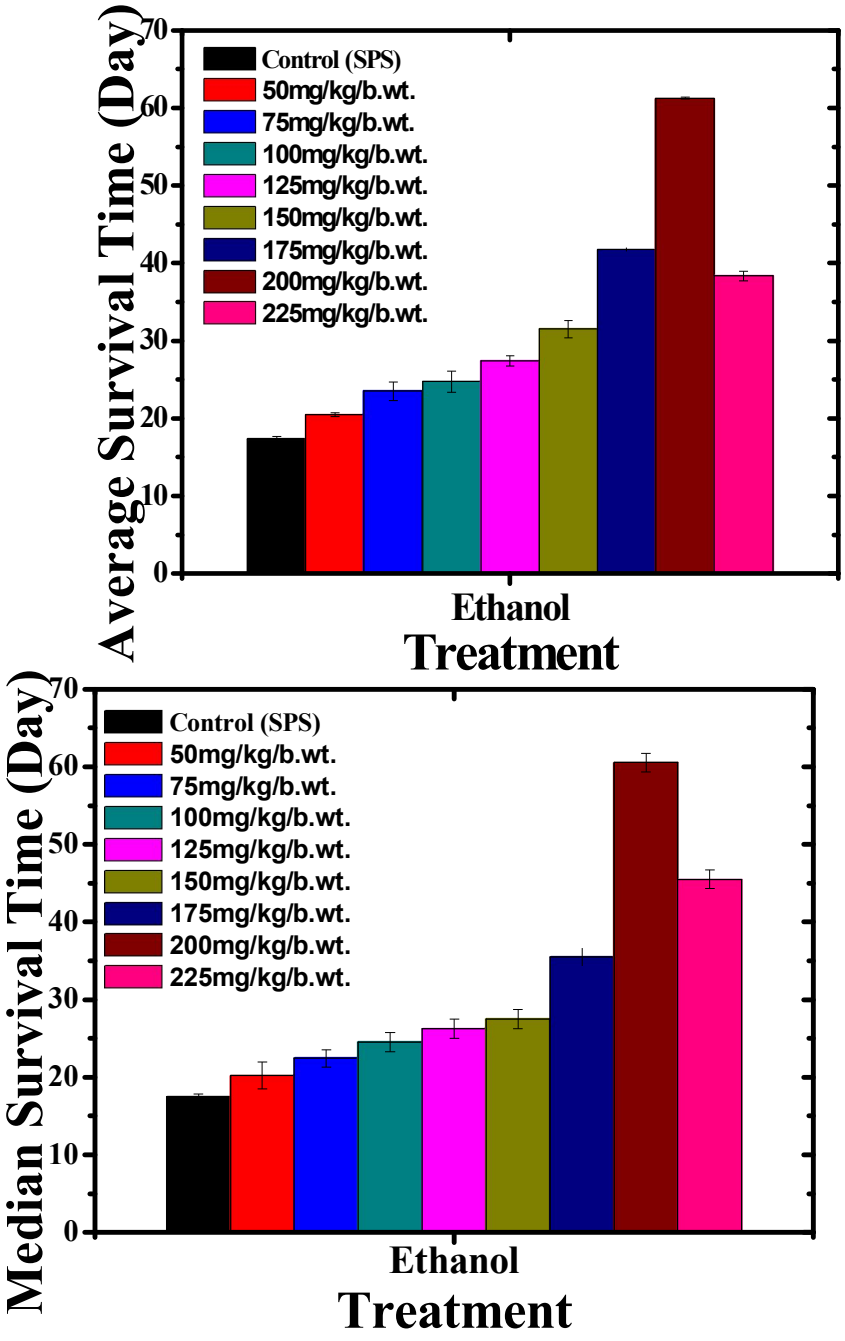


Figure 3: Effect of ethanolic extract of *Colocasia gigantea* in Dalton's lymphoma ascites bearing mice on the tumor response assessment based on average survival time (AST) and median survival time (MST). N=10.

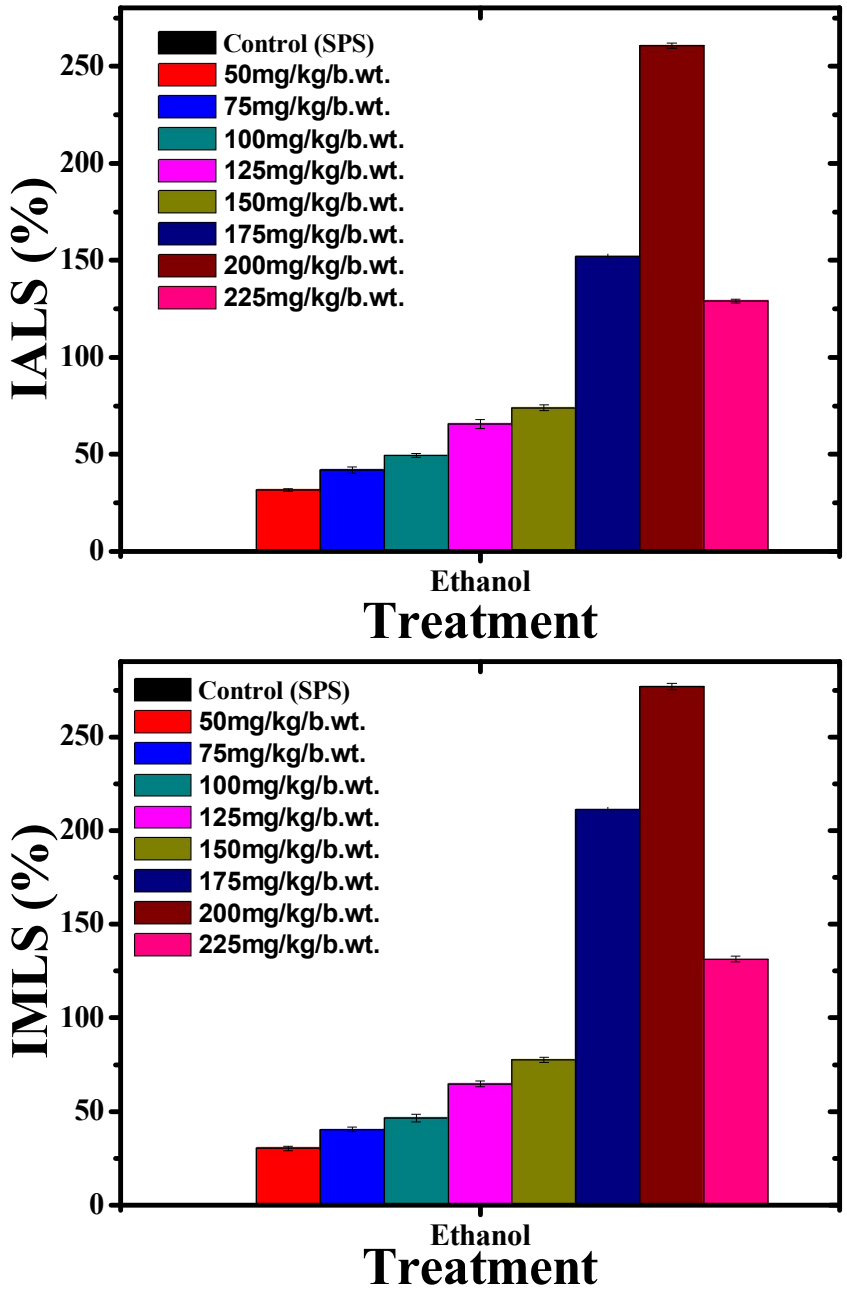


Figure 4: Effect of ethanolic extract of *Colocasia gigantea* in Dalton's lymphoma ascites bearing mice on the tumor response assessment based on increase in average life span (% IALS) and increase in mean life span (%IMLS). N=10.

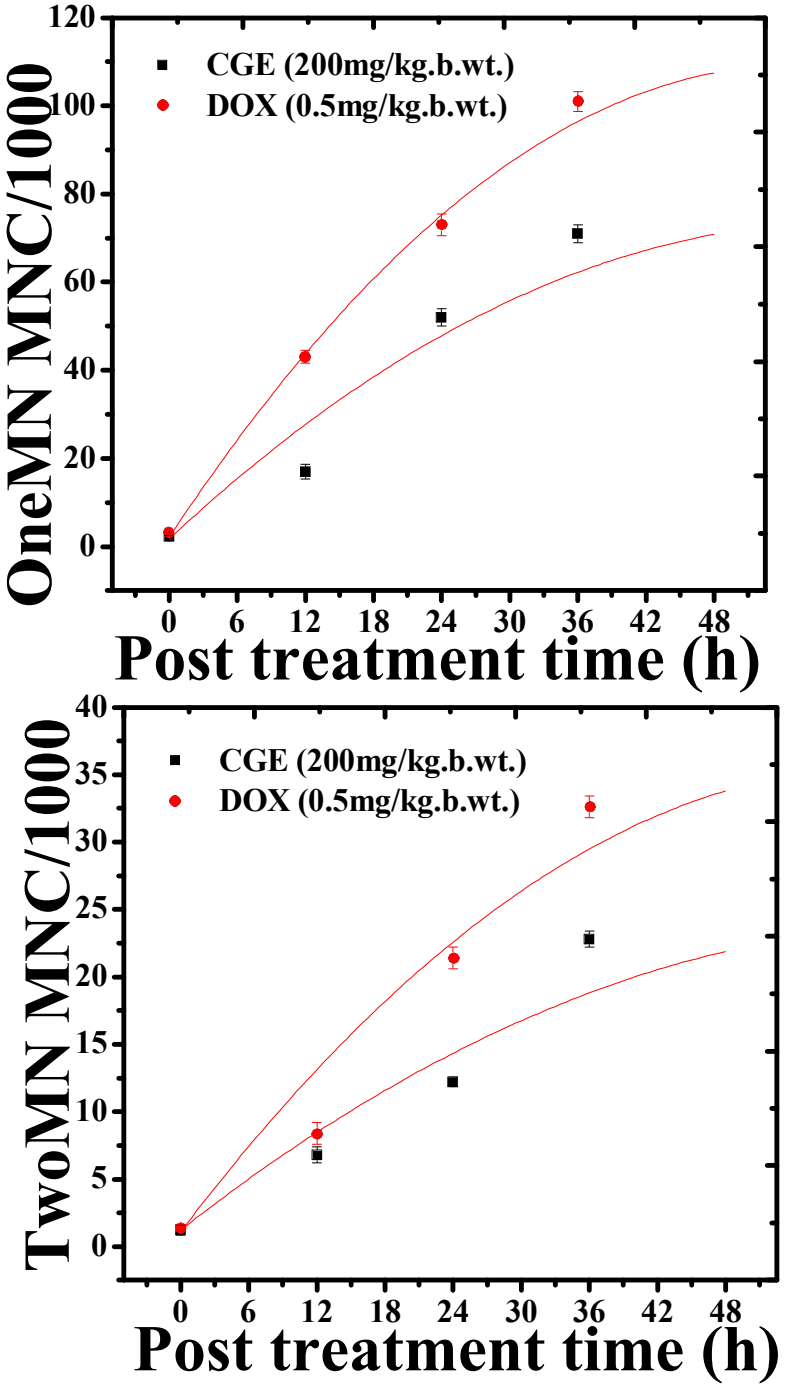


Figure 5(a): Frequency of micronucleated mononucleate cells in Dalton's lymphoma ascites bearing mice treated with 200mg/kg b.wt. ethanol extract of *Colocasia gigantea* (CGE) or 0.5mg/kg b.wt. doxorubicin (DOX) at different post treatment times. Above left: One micronucleus in mononucleated cells. Above right: Two micronucleus in mononucleated cells. N=5.

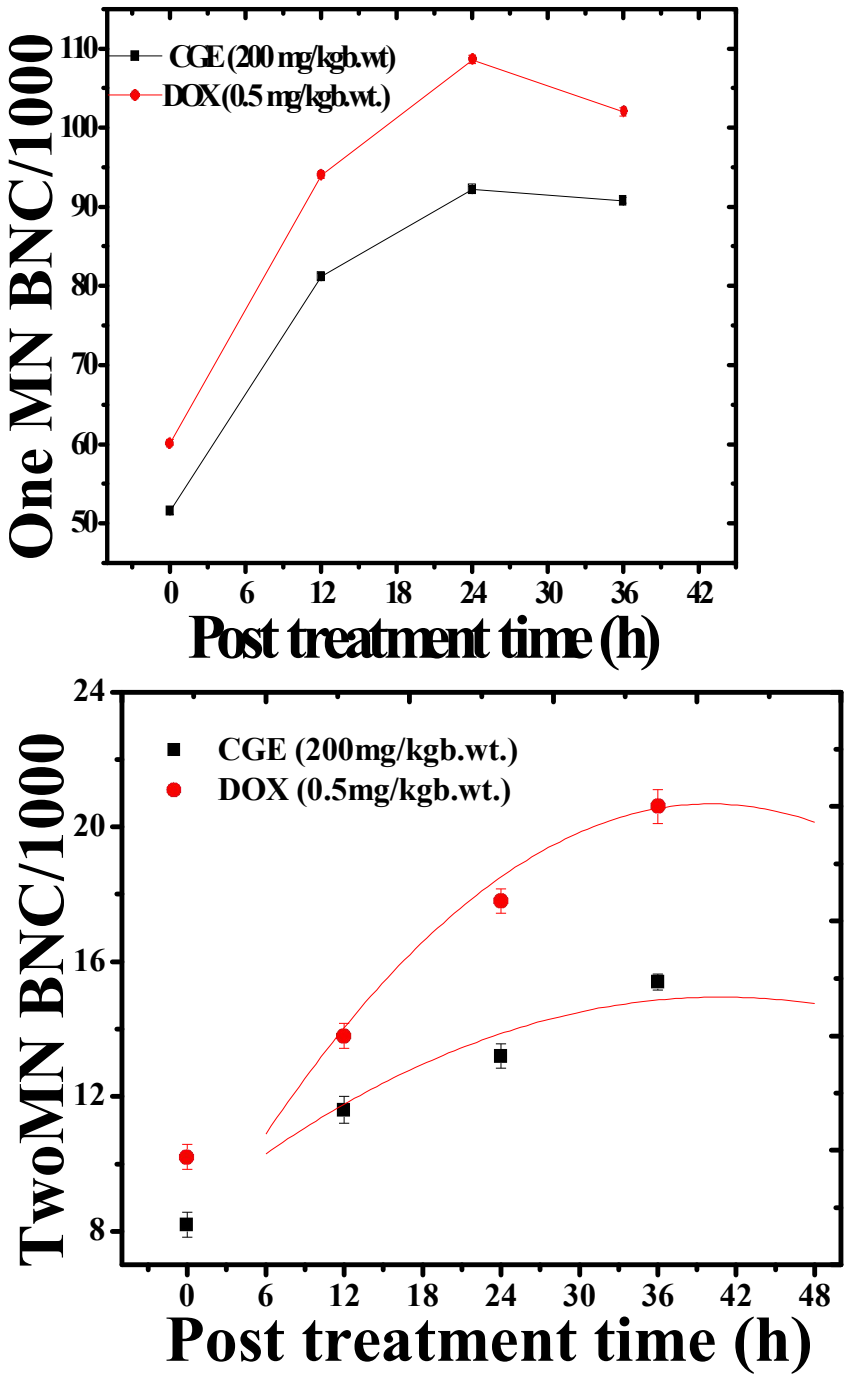


Figure 5(b): Frequency of micronucleated binucleate cells in Dalton's lymphoma ascites bearing mice treated with 200 mg/kg b. wt. ethanol extract of *Colocasia gigantea* (CGE) or 0.5mg/kg b. wt. doxorubicin (DOX) at different post treatment times. Above left: One micronucleus in binucleated cells. Above right: Two micronucleus in binucleated cells. N=5.

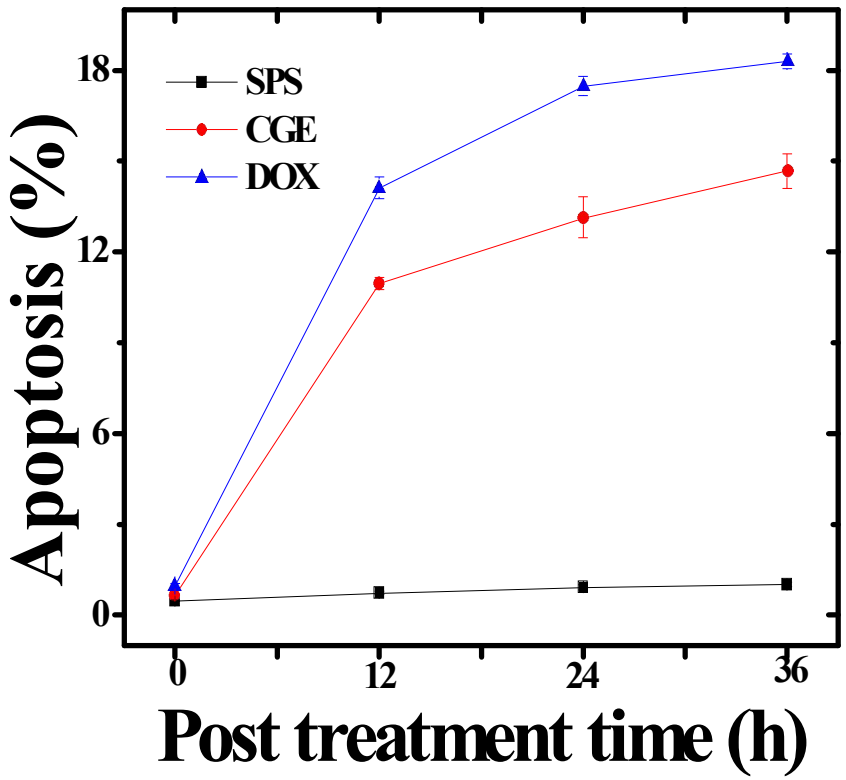


Figure 6: Apoptotic index in Dalton's lymphomas ascites bearing mice treated with 200mg/kg b.wt. ethanolic extract of *Colocasia gigantea* (CGE) or 0.5mg/kg b.wt. doxorubicin (DOX) at different post treatment times. N=5.

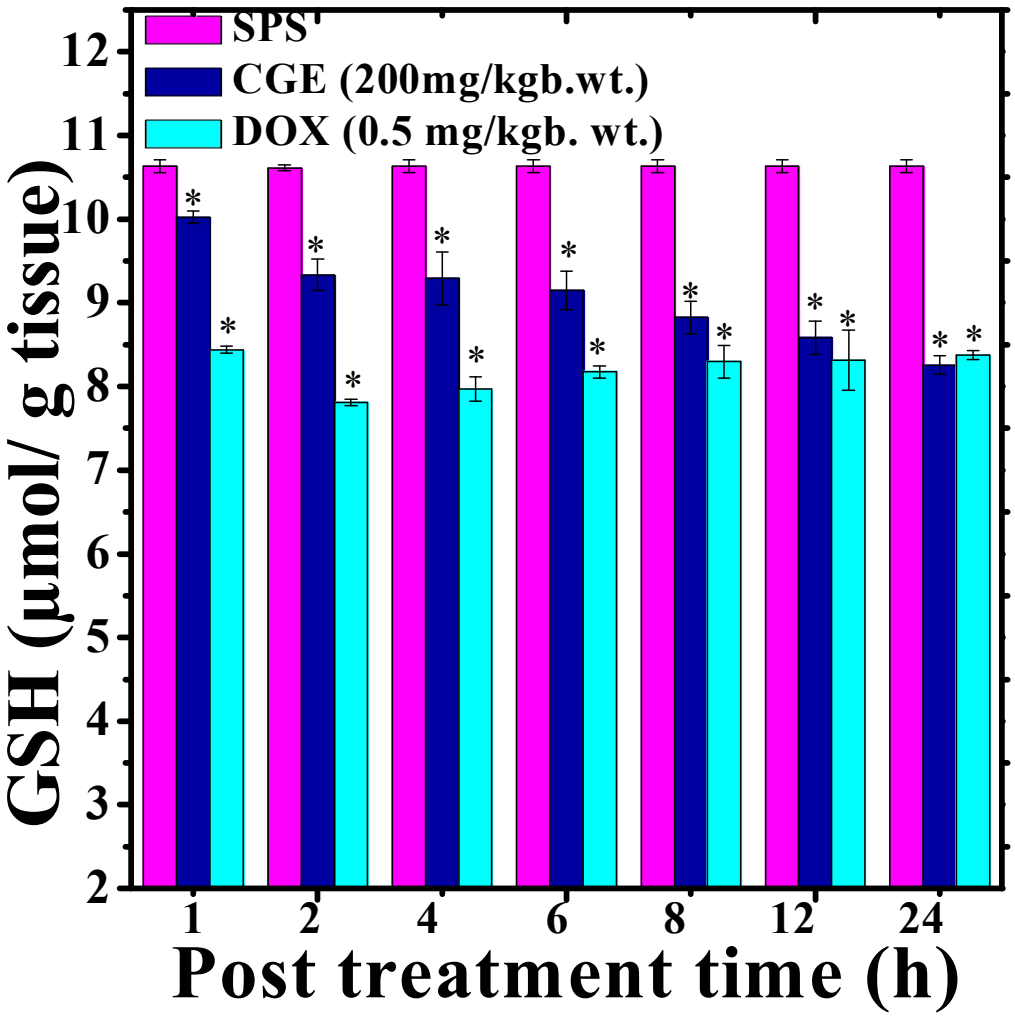


Figure 7: Alteration in the Glutathione contents in mice bearing Dalton's lymphoma ascites treated with 200 mg/kg b.wt. of *Colocasia gigantea* extract (CGE) or doxorubicin (DOX). N=10.

***p<0.001 when treatment are compared with control (SPS) group.**
No symbol= no significance.

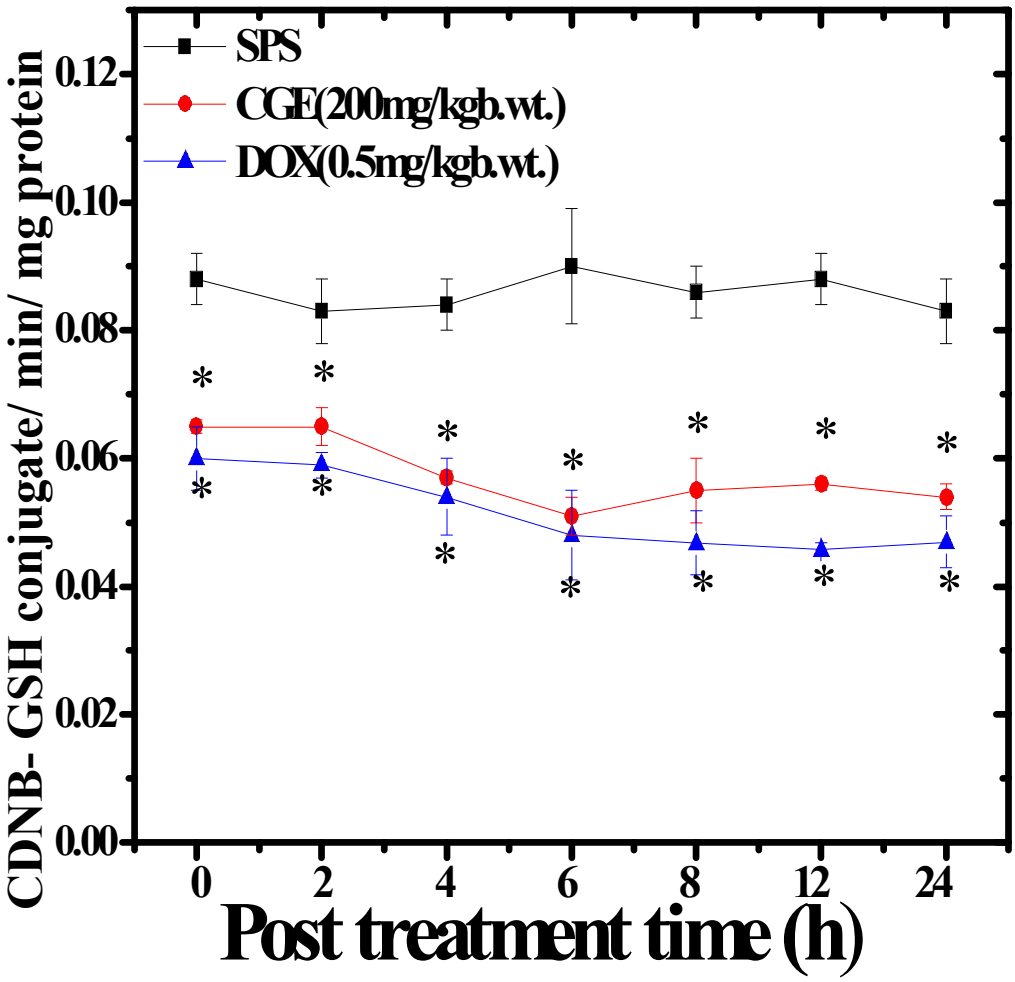


Figure 8: Alterations in the glutathione-s-transferase (GST) activity in Dalton's lymphoma ascites bearing mice treated with 200 mg/ kg b. wt. of *Colocasia gigantea* (CGE) or doxorubicin (DOX). N=10.

***p<0.001 when treatment are compared with control (SPS) group.**
No symbol= no significance.

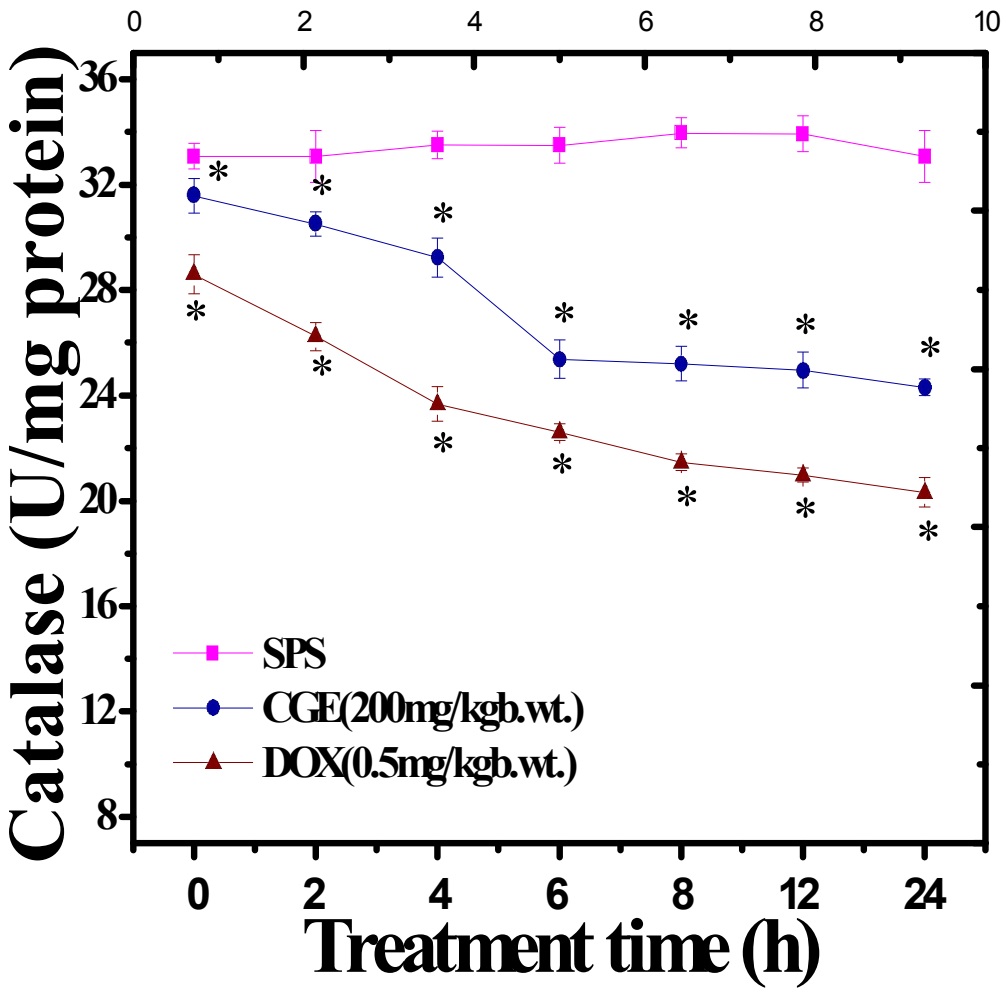


Figure 9: Alterations in the catalase (CAT) activity of Dalton's lymphoma ascites bearing mice treated with 200 mg/ kg b. wt. of *Colocasia gigantea* (CGE) or doxorubicin (DOX). N=10.

***p<0.001 when treatment are compared with control (SPS) group.**
No symbol= no significance.

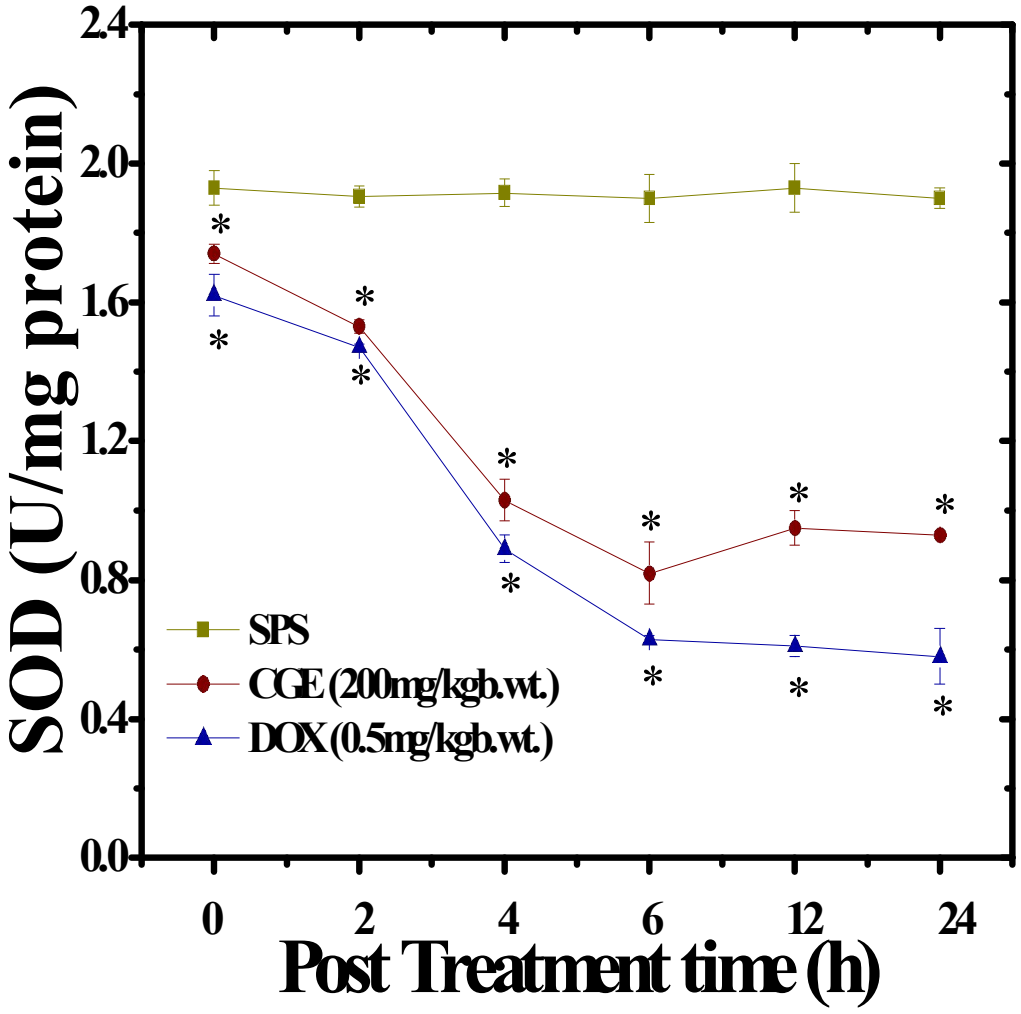


Figure 10: Alterations in the Superoxide dismutase (SOD) activity of Dalton's lymphoma ascites bearing mice treated with 200 mg/ kg body weight of *Colocasia gigantea* (CGE) or doxorubicin (DOX). N=10

***p<0.001 when treatment are compared with control (SPS) group.**
No symbol= no significance.

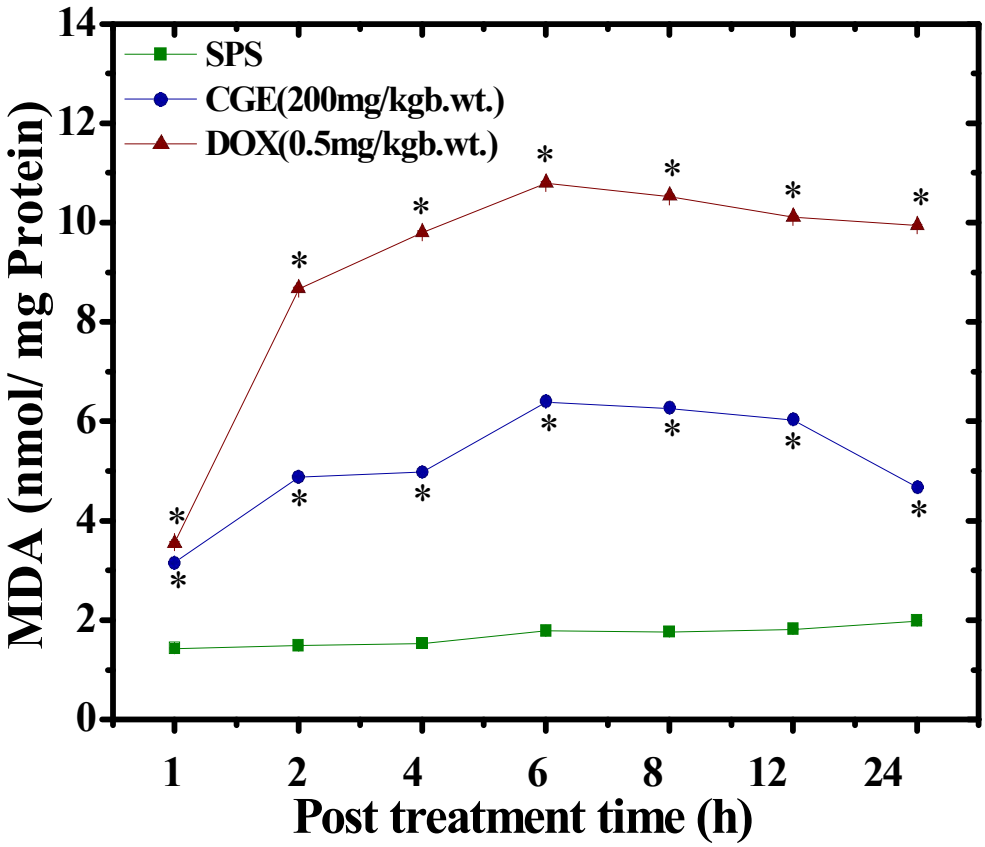


Figure 11: Alterations in the lipid peroxidation (LOO) in the Dalton's lymphoma ascites bearing mice treated with 200 mg/ kg b. wt. of *Colocasia gigantea* (CGE) or doxorubicin (DOX). N=10

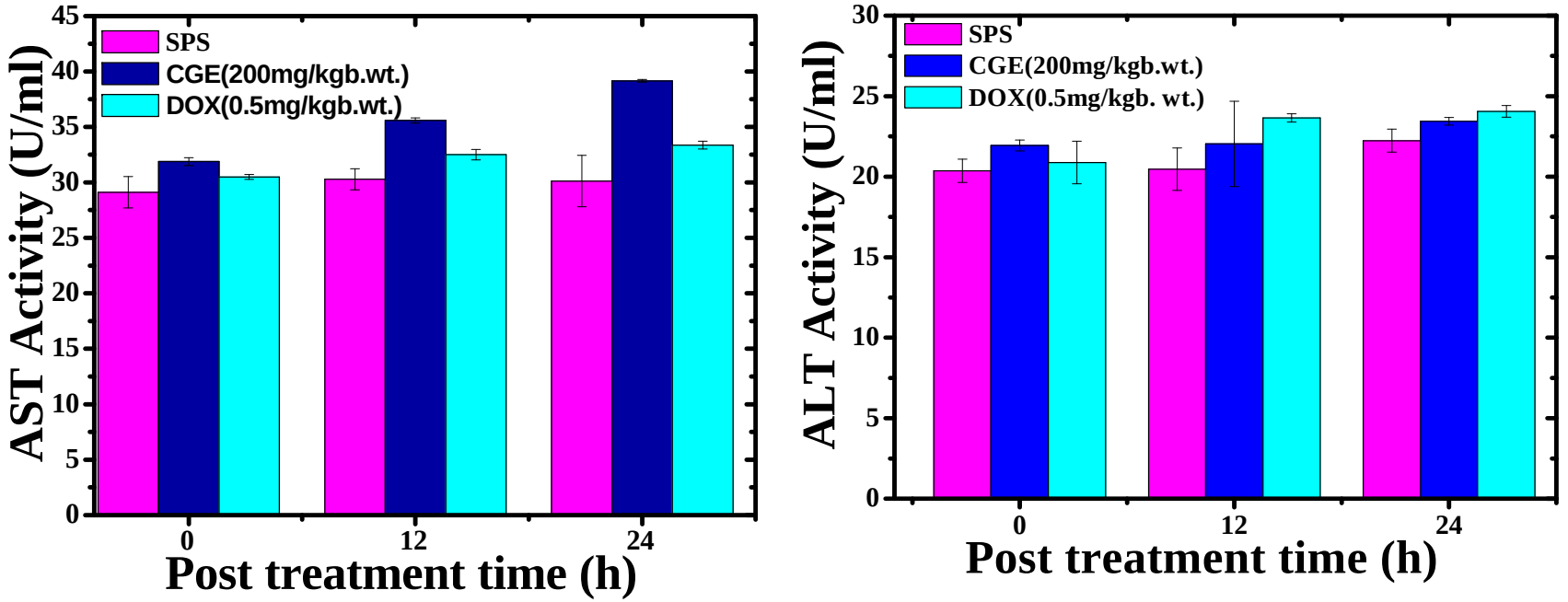


Figure12(a): The alteration in the liver function by 200 mg/kg b.wt. of *Colocasia gigantea* (CGE) or 0.5 mg/kg b.wt. of doxorubicin DOX in Dalton's lymphoma ascites bearing mice. N=10

P<0.05 when treatment are compared with control (SPS) group.

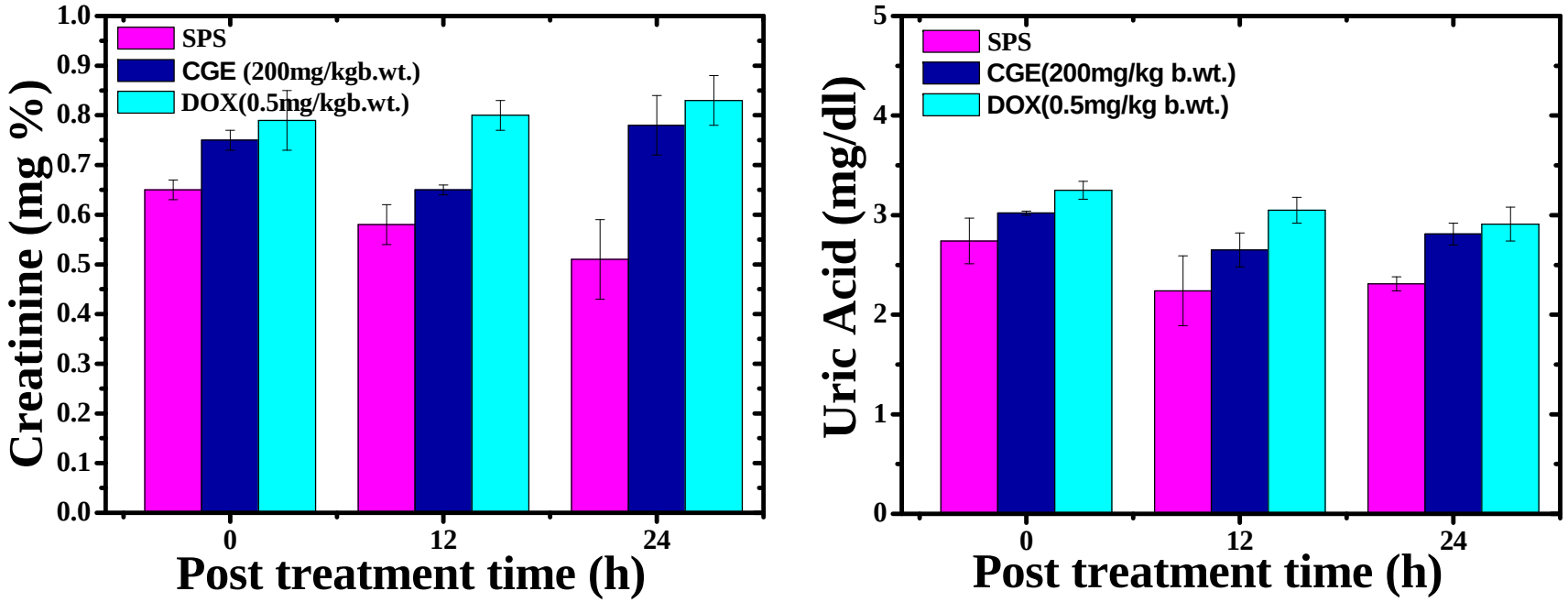
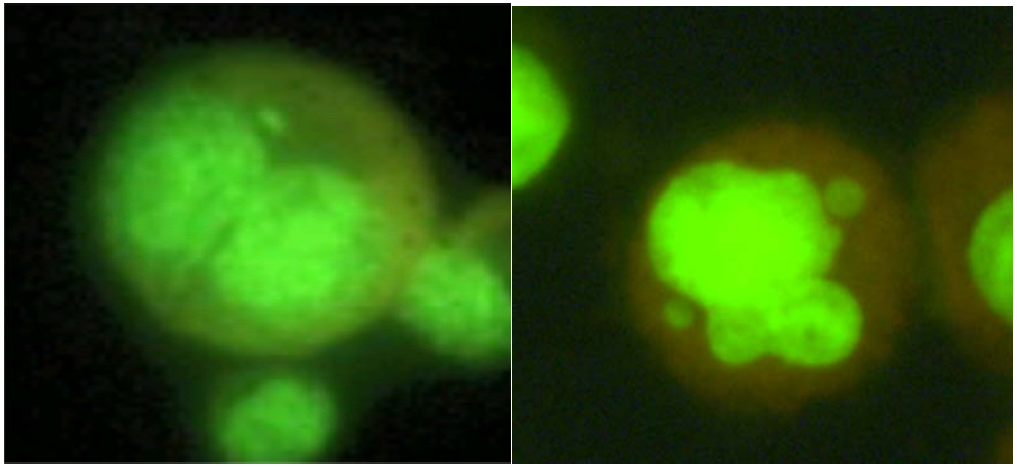


Figure 12(b): The alteration in the kidney function by 200 mg/kg b.wt. of *Colocasia gigantea* (CGE) or 0.5 mg/kg b.wt. of doxorubicin DOX in Dalton's lymphoma ascites bearing mice. N=10

P<0.05 when treatment are compared with control (SPS) group.



(a) Mononucleated cell **(b) Binucleated cell**

Figure 13: Micronuclei images of Dalton's lymphoma cells treated with *Colocasia gigantea*

(a) Mononucleated cell (b) Binucleated cell

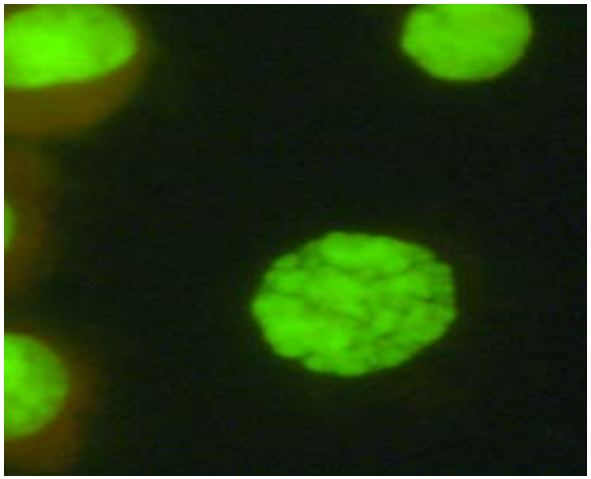


Figure 14: Photomicrograph of Dalton's Lymphoma cells showing apoptosis after treatment with *Colocasia gigantea*.

(a) 100 mg CGE **(b) 200 mg CGE** **(c) 300 mg CGE**

Figure 15: Clonogenic Assay of HeLa cells treated with various concentrations of *Colocasia gigantea* (a) 100 mg CGE, (b) 200 mg CGE and (c) 300 mg CGE.

CHAPTER 6

SUMMARY AND CONCLUSIONS

Chapter 6

INTRODUCTION

Cancer is a Latin word, which literally means crab and it is known as *karkinos* in the Greek language, which also means crab. Cancer is assumed to be older than humans as it has been identified in the bones of dinosaurs from the Jurassic periods (Greaves, 2000). The earliest evidence of human cancer found till date is a metastatic carcinoma in a young man from ancient Nubia which dated back to 1200 BC (Binder *et al.,* 2014). Hippocrates, the father of modern medicine observed that the blood vessels around malignant tumors looked like the claws of a crab and hence he coined the term cancer to such tumors for the first time. Humans have known cancer as a malignant disease as early as Ramayan and Egyptian (3000 to 1500 BC) periods. The Ramayana and Egyptian Papyrus even described the cure for cancer (Woelfer, 1881; Breasted, 1930; Ebbell, 1937).

Cancer is a group of diseases that comprises mutation or dynamic changes in the genome of the cell producing proteins that disturb the normal cellular balance leading to the uncontrolled proliferation of cells (Bishop and Weinberg, 1996; Hejmadi, 2010; American Cancer Society, 2015). In normal condition, the cells enter the active proliferative phase only after receiving the mitogenic growth signals, and cannot multiply in the absence of these signals. However, these stimulatory signals are deregulated in cancer cells and therefore cancer cells are able to proliferate even in the absence of these signals (Hanahan and Weinberg, 2000). Cancer cells have the ability to produce their own growth factors mimicking the normal growth factors which make them independent of the normal growth factors (Fedi *et al.,* 1997).

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Cancer is one of the leading cause of death in the developed world, which is second to cardiovascular diseases (Siegel *et al*., 2016). Cancer is classified based on the part of the body in which it begins, and by its appearance under a microscope. Different types of cancer vary in their growth rates, patterns of spread, and responses to different types of treatment. The life style and diet also play a major role in the causation of cancer (WHO, 2003). There are many factors that can cause cancer either directly or indirectly which can be broadly classified as environmental, lifestyle and behavioural exposures (Steward and Wild, 2014). Common environmental factors that contribute to cancer death include tobacco (according to one estimate, accounting for 25–30% of deaths), obesity (30–35%), infections (15–20%), radiation (both ionizing and non-ionizing, up to 10%), lack of physical activity, and environmental pollutants (Anand *et al.,* 2008).

Cancer treatment usually involves the combination or single use of surgery, radiotherapy or systemic therapy. Earlier, stages of cancer can be cured with surgery alone; however, systemic therapy is the only potion once the disease has metastasized since delivery through the bloodstream is required for cancer cells at different sites. The systemic therapy includes hormonal therapy, targeted therapy and chemotherapy (Caley and Jones, 2012). Different types of cancer treatments have their limitations. During surgery, some cancer cells may be missed while removing the surrounding tissues so as to clear all the cancer cells and can lead to loss or reduced functioning of the organs. Besides, it can be used for the treatment of later stages of cancer the has already metastasized (Greene *et al.,* 2002).

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Chemotherapy also has its own limitations as it does not specifically target tumor cells. Since the chemotherapeutic agents hamper cell division or inhibit enzymes involved in DNA replication or metabolism, they also damage the normal dividing cells especially the rapidly regenerating tissues, such as those of bone marrow, gut mucosa and hair follicles (Wu *et al.,* 2008). Therefore, it is desirable for the search for new drugs, which are less toxic and do not produce undesires changes in the normal cells (Newman and Cragg, 2014).

Plants have been the major source for several drugs and it is well known that 75% of the modern chemotherapeutic drugs have their origin in plants or natural products (Cragg and Newman, 2013; Harvey *et al.,* 2015). Several phytoceuticals have been screened for their anticancer activities earlier. *Alsotnis scholaris, Aegle marmelos, Aphanmixis polystychya, Solanum khasianum, Tinospora cardiofolia* have been found to possess anticancer activity in different preclinical systems (Jagetia *et al.,* 1998; Jagetia and Baliga; 2005; Jagetia *et al.,* 2005; Jagetia and Venkatesha , 2012; Rosangkima and Jagetia, 2015). Extracts from *Urtica membranacea, Artemesia monosperma* and *Origanum dayi* post have also been reported to exert anticancer activity (Solowey *et al.,* 2014). *Artocarpus obtusus, Blumea balsamifera, Boerhaavia diffusa, Calotropis procera, Citrus maxima, Emblica officinalis, Saxifraga stolonifera, Vitex negundo, Withania somnifera and Zingiber officinale* have also been found to possess anticancer activity under different conditions (Merina *et al.,* 2012). Therefore, plants and natural products plays a major role in screening and developing new nontoxic molecules including drugs for cancer treatment.

Chapter 6

AIM OF THE STUDY

The control of cancer is one of the leading causes of death worldwide. Conventional therapies cause serious side effects and, at best, merely extend the patient's lifespan by a few years. Better cancer treatments with milder side effects are desperately needed. There is a need to utilise alternative concepts or approaches to prevent the cancer (Reddy *et al.,* 2003). Interestingly, both laboratory experiments andclinical trials have demonstrated that when combined with chemotherapy, herbal medicines could raise the efficacy level and lower toxic reactions. These facts raised the feasibility of the combination of herbal medicine and chemotherapy (Ruan *et al.,* 2006).

Herbal medicines are usually less toxic compared to the pure synthetic compounds due their composite nature and presence of other chemicals. Several plants have provided valuable antineoplastic compounds. However, these compounds in pure synthetic form proved toxic to normal cells. Therefore, there is a need to screen new pharmacological products, which are less toxic, highly effective and economically cheaper in treating the neoplastic disorders. Research in traditional medicine has led to the development of many modern medicines. Therefore the use of herbal medicines may be helpful to treat cancer.

Colocasia gigantea (family: Araceae) also called giant elephant ear or Indian taro grows up to a height of 1.5-3 m with a whorl of large leaves at its apex. It bears a large, fibrous, and inedible corm. *Colocasia gigantea* grows commonly in India, Thailand and other Southeast Asian countries. The leaf stalks are used as a vegetable in some areas in, India, South East Asia and Japan. In India the tubers are cooked

and used as a vegetable (Kay, 1987). In Thailand, *C. gigantea* is considered as a minor food crop and is mainly utilized as a stem vegetable. The stalks are often used for making homemade Thai food called "Bon curry". In the Pacific islands, the tubers are cooked and eaten as a starch (Manner, 2011). In Thai traditional medicine, *C. gigantea* tuber is heated over a fire and is used to reduce "internal heat" (fever) and also for the treatment of drowsiness. The fresh or dried tubers mixed with honey are used in the treatment of phlegm in northern Thailand. Fresh tubers have been shown to ameliorate stomach problems, combat infection, and accelerate the healing of wounds. In Fiji, the locals make use of either boiled or baked breadfruit or tubers of taro as slices along with roasted pig (Muralidharan, 1992). Uses of taro in native recipes along with its medicinal uses in treatment of constipation and tuberculosis in Hawaii (Kokua1977). Pressed juice of petiole of taro is highly cystic and is even said to arrest arterial haemorrhage (Drury, 1873). Medicinal value of taro (But *et al.,* 1980) in China. Nutritionally, taro is very similar to tannia. Its starch contains 17.5% amylose and the rest amylopectin. Starch grain is very small and the size ranges from 1-4 μ. It is rich in most of the essential amino acids (Rao and Polacchi, 1972) and hence is considered to be a good leafy vegetable. It is reported that 100 g of taro tuber contains 73.1 g moisture, 3 g protein, 0.1 g fat, 1.7 g minerals, 22.1 g carbohydrates, 0.04 g calcium, 0.14 g phosphorus, 2.1 mg iron, 80 IU Vitamin B and trace of Vitamin C (Shanmugavelu, 1989). Therefore, the present study was carried out to obtain an insight into the anticancer activity of *Colocasia gigantea* extracts *in vitro* and *in vivo* .

Chapter 6

CHAPTER 1

This chapter introduces the subject and discusses about cancer, its history, types, causes, the different stages of cancer, various treatment regimens used to treat cancer and also lists out the aims and objectives of the study.

CHAPTER 2

This chapter gives an account of phytochemical screening TLC profile of various extracts of *Colocasia gigantea*. The non-infected rhizomes of *Colocasia gigantea* were collected, shade dried and powdered. The powdered rhizomes were sequentially extracted in petroleum ether, chloroform, ethanol and distilled water using Soxhlet apparatus and the liquid extracts were concentrated with rotary evaporator and stored at -70°C until further use. Qualitative phytochemical analysis was carried out on all the extracts except petroleum ether which was discarded. *Colocasia gigantea* was found to contain alkaloids, carbohydrates, phytosterols, saponins, tannins, cardiac glycosides and flavonoid. The TLC study also showed the presence of different components.

CHAPTER 3

This chapter describes the in vitro antioxidant activity of the different extracts of *Colocasia gigantea.* Free radicals are necessary evils as they are produced during normal metabolism of the body as well as a defence against the pathogenic attack. However, excess of free radical generation produce oxidative stress, inflammation and various diseases and any agent that can reduce the oxidative stress will be useful in treating the oxidative stress related disorders. Therefore, it is necessary to investigate the free radical scavenging activity of different extracts of *Colocasia*

gigantea in vitro. The antioxidant activity of various extracts was evaluated by their ability to inhibit the generation of DPPH, hydroxyl ($^{\bullet}OH$), superoxide ($O_2^{\bullet-}$), $ABTS^{\bullet+}$and nitric oxide ($NO^{\bullet}$) radicals in vitro. Total flavonoid and the total phenol contents were also determined to understand their role in free radical scavenging. The chloroform, ethanol, and aqueous extracts of *Colocasia gigantea* showed a concentration dependent inhibition in DPPH, $^{\bullet}OH$, $O_2^{\bullet-}$, $ABTS^{\bullet+}$ and $NO^{\bullet}$ radical generation. The *C. gigantea* scavenged DPPH, $^{\bullet}OH$, $O_2^{\bullet-}$, $ABTS^{\bullet+}$ and $NO^{\bullet}$ radicals in a concentration dependent manner and this activity may be due the presence of various polyphenols and flavonoids in its rhizomes.

CHAPTER 4

This chapter deals with the cytotoxic effects of the ethanol extract of *Colocasia gigantea* (CGE) in HeLa and V79 cells by MTT assay, which showed a concentrations dependent rise in the cytotoxicity. The maximum cytotoxicity was observed at 300 and 200 µg/ml ethanol extract of *Colocasia gigantea* for HeLa and V79 cells, respectively. Assessment of treatment duration revealed that cytotoxic effect of ethanol extract of *Colocasia gigantea* was marginal increased with treatment duration. Treatment of HeLa cells with different concentrations of ethanol extract of *Colocasia gigantea* reduced the clonogenicity of cells in a concentration dependent manner, which reached a nadir at 300µg/ml. To understand the biochemical mechanism of action, the HeLa cells were treated with different concentrations of ethanol extract of *Colocasia gigantea* and contents of glutathione and activities of the glutathione-s-transferase and catalase and lipid peroxidation were determined. The ethanol extract of *Colocasia gigantea* reduced the glutathione concentration and activities of the glutathione-s-transferase and catalase in a

concentration and time dependent manner and greatest reduction was observed at 6 h post treatment, whereas lipid peroxidation increased in a concentration and time dependent manner. The ethanol extract of *Colocasia gigantea* induced cytotoxicity and reduced the reproductive integrity of HeLa cells. The cytotoxicity of ethanol extract of *Colocasia gigantea* may be due to elevated lipid peroxidation and reduced concentration of glutathione and glutathione-s-transferase and catalase activities.

CHAPTER 5

The chapter gives an account of the acute toxicity in normal non-tumour bearing mice administered with different doses of various extracts of *Colocasia gigantea* orally or and intraperitoneally. The oral administration of chloroform, ethanol and aqueous extracts of *Colocasia gigantea* were non-toxic up to 2 g/kg body weight. The intraperitoneal administration of different extracts of *Colocasia gigantea* exhibited toxic effect and the LD50 for ethanol extract was found to be 0.2 g/kgb.wt. whereas it was 0.15 g/kgb.wt. for chloroform and aqueous extracts, respectively. The determination of anticancer activity by intraperitoneal administration of 0, 50, 75, 100, 125, 150, 175, 200 or 225 mg/kg b.wt. of ethanol extract to Dalton's lymphoma transplanted mice resulted in a dose dependent rise in tumour regression and increase in the average survival as well as median survival time. The maximum tumour free survivors were observed at 200 mg/kg b.wt. of ethanol extract and further experiments were carried out using this dose. The evaluation of micronuclei showed that the ethanol extract *Colocasia gigantea* increased the frequency of micronucleated mononucleate cells as well as micronucleated binucleate cells in a time dependent manner and their frequencies

were maximum at 36 h post-treatment. Similarly ethanol extract *Colocasia gigantea* increased the apoptosis index also increased in a time dependent manner and the highest apoptosis was observed at 36 h post treatment. The biochemical studies revealed a significant decline in the glutathione, glutathione-s-transferase, superoxide dismutase and catalase accompanied by elevated lipid peroxidation. The safety of ethanol extract of 200 mg *Colocasia gigantea* was ascertained by evaluating aspartic acid transaminase, and alanine aminotransferase, creatinine and uric acid at different post treatment times in Dalton's lymphoma bearing mice liver and kidney. These parameters did not show any significant alteration and they were within the normal range. The ethanol extract of *Colocasia gigantea* did not show any toxicity orally however, intraperitoneal administration did exert toxic effects and it also induced anticancer activity in tumour cells by increasing tumour free survivors. The cytotoxic effect of ethanol extract may be due to induction of DNA damage in the form of micronuclei and apoptosis and reduction in glutathione, glutathione-s-transferase, superoxide dismutase and catalase accompanied by a rise in the lipid peroxidation.

Chapter 6

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RESEARCH PUBLICATIONS

1. **Devi N. Bonika, Jagetia GC. Antitumor activity of different extracts of *Colocasia gigantea* in Dalton's lymphoma transplanted Swiss Albino mice. International Research Journal of Pharmaceutical and Biosciences (IRJPBS); 2017; 4 (4):52-82.**

2. **Devi N. Bonika, Jagetia GC. Anticancer activity of *Colocasia gigantea* (Blume) Hook. f. in cultured cell lines. International Journal of Current Engineering and Scientific Research (IJCES) 2017; 4(9): 2393-8374.**

3. **Devi N. Bonika, Jagetia GC. Free radical scavenging and antioxidant potential of different extracts of *Colocasia gigantea* (Blume) Hook. F. *In vitro*. International Research Journal of Pharmacy, 2017, 8(10):72-81.**

BOOK IN CHAPTER

1. **Effects of Methanolic Extract of *Emblica officinalis* against Isoproterenol- Induced Cardiotoxicity in Rats. Science and Technology for Shaping the Future of Mizoram. (13^{th} -14^{th} October 2016) Allied Publishers Pvt. Ltd., New Delhi (India).**

2. **Phytochemical Analysis and Antioxidant properties of extracts of the rhizomes of *Colocasia gigantea* (Blume) Hook. *f.* Biodiversity, Conservation and Utilization of Natural Resources with reference to Northeast India. (30^{th} – 31^{st} March 2017) Panima Publishing Corporation, New Delhi, Bangalore (India).**

3. **Acute Toxicity Study of Various Extracts of *Colocasia gigantea* (Blume) Hook. *f.* on Swiss Albino mice. Natural Resources Management for Sustainable Development and Rural Livelihoods. Volume 3. (26^{th} – 28^{th} October 2017) Today and tomorrow's Printers and Publishers, New Delhi (India).**

SEMINARS PRESENTED

1. **Presented a poster presentation in the National Conference on "Science and Technology for Shaping the Future of Mizoram" held on 13th -14th October 2016 at the Auditorium, Mizoram University, organized by Mizoram Science Congress 2016 Organising Committee, sponsored by NEC, DST (SERB) and MISTIC.**

2. **Presented a research paper in the National Level Seminar on "Biodiversity, Conservation and Utilization of Natural Resources with reference to Northeast India (BCUNRNEI)" held on 30th – 31st March 2017, organized by the Department of Botany, Mizoram University Aizawl 796004, sponsored by National Biodiversity Authority, India.**

3. **Presented a poster presentation in the International Conference on "Natural Resources Management for Sustainable Development and Rural Livelihoods" held on 26th -28th October 2017 hosted by the Department of Geography and Resource Management, School of Earth Science and Natural Resource Management, Mizoram University at Academic and Seminar Complex, Mizoram University.**

SEMINARS AND WORKSHOP PARTICIPATED

1. **Participated in the UGC Sponsored 3 weeks Programme on "Interaction Programme for Ph.D. Scholars" organized by UGC Academic Staff College, Mizoram University Aizawl 796004, sponsored by University Grants Commission held during 23rd September – 12th October, 2013.**
2. **Participated in the One Week Workshop on "Applied Statistics" organized by UGC Academic Staff College, Mizoram University Aizawl 796004, sponsored by University Grants Commission held during 23rd September – 28th September, 2013.**
3. **Participated in "Science Communication Workshop (sciComm 101)" organized by The Welcome Trust/DBT India Alliance held on 6rd June 2017.**
4. **Participated in the Workshop on "Mechanism of Adaptation in the Temporal Environment" organized by Department of Zoology, Mizoram University Aizawl 796004, held on 23th May 2017.**
5. **Participated in "Workshop on Capacity Building in Effective Management of Intellectual Property rights (IPRS) in Biotechnology by Universities and Research Institutes in Mizoram" organized by Biotech Consortium India Limited (BCIL), New Delhi, sponsored by department of Biotechnology, Government of India held on 27th – 28th August, 2014.**
6. **Participated in "105th Indian Science Congress" held at Manipur University, Imphal from 16th -20th March 2018.**

ANTICANCER ACTIVITY OF COLOCASIA GIGANTEA (BLUME) HOOK. f. IN CULTURED CELL LINES

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ABSTRACT

Evaluation of cytotoxic effects of the ethanol extract of *Colocasia gigantea* (CGE) in *HeLa* and *V79* cells by MTT assay showed a concentrations dependent rise in the cytotoxicity. The maximum cytotoxicity was observed at 300 and 200 µg/ml ethanol extract of *Colocasia gigantea* for HeLa and V79 cells, respectively. Assessment of treatment duration revealed that cytotoxic effect of ethanol extract of *Colocasia gigantea* was marginal increased with treatment duration. Treatment of *HeLa* cells with different concentrations of ethanol extract of *Colocasia gigantea* reduced the clonogenicity of cells in a concentration dependent manner, which reached a nadir at 300 µg/ml. To understand the biochemical mechanism of action, the *HeLa* cells were treated with different concentrations of ethanol extract of *Colocasia gigantea* and contents of glutathione and activities of the glutathione-s-transferase and catalase and lipid peroxidation were determined. The ethanol extract of *Colocasia gigantea* reduced the glutathione concentration and activities of the glutathione-s-transferase and catalase in a concentration and time dependent manner and greatest reduction was observed at 6 h post treatment, whereas lipid peroxidation increased in a concentration and time dependent manner. The ethanol extract of *Colocasia gigantea* induced cytotoxicity and reduced the reproductive integrity of HeLa cells. The cytotoxicity of ethanol extract of *Colocasia gigantean* may be due to elevated lipid peroxidation and reduced concentration of glutathione and glutathione-s-transferase and catalase activities.

Key words: Colocasia gigantea, MTT, clonogenic, GSH, GST and catalase.

INTRODUCTION

Despite availability of state or art treatment regimen cancer still remains one of the leading causes of death in both the developed as well as in the under developed countries [1]. It is estimated that one in every eighth deaths is due to cancer [2]. Cancer is a multifaceted disease and with improving health facilities and increased life span more cancer cases are coming to light than ever before. This has also increased the mortality rates and cancer deaths are of major concern globally[3]. Apart from many synthetic drugs the natural products have also immensely contributed to the paraphernalia of chemotherapeutic drugs. The fact is that 80% of the global population still depend on plants and natural product for their healthcare proves the importance of plants as a major source of medicine. The one third of the all drugs approved by Federal Drug Administration, USA has been natural products [4]. This reemphasizes the importance of plants and natural products in healthcare and new drug discovery. The evaluation of natural products could provide a new breakthrough in cancer treatment and new technologies are being explored for obtaining novel compounds from biodiversity of nature. The pharmaceutical industry has a continuing need to find new and better chemical compounds to develop as drugs for human healthcare [5]. Many drugs used for the treatment of different diseases including cancer are obtained from natural products [6] and plants provide a major platform for design and new drug discovery. About 75 % of the registered small anticancer molecules since the 1940s have drug discover form the complex secondary metabolites

synthesized by plants. Therefore it is necessary to screen diverse plants for their anticancer activity in the hope that there will be a time one it may be possible to come across some biomolecules that will treat cancer effectively with lesser adverse side effects. *Colocasia gigantea* (family Araceae), also known as Giant Elephant ear or Indian taro, is a stemless plant producing large leaves with underground rhizomes. The rhizomes and the stalks are eaten as a vegetable and the leaves are eaten raw with pomegranate in India. In Thailand, *C. gigantea* tubers are heated over fire and consumed as a medicine[7]. It is used to treat drowsiness and to reduce internal heat. The tuber reduces stomach problems, cures infection and heals wounds. Fresh or dries tubers are being used in the treatment of phlegm along with honey [8]. It is also used in the treatment of tuberculosis and constipation in Hawai [9]. Juice of taro are said to arrest arterial hemorrhage[10]. The information regarding the anticancer activity of *Colocasia gigantea* is lacking and it is used as a diet, which indicates that if is found to kill cancerous cells it may be a useful paradigm to fight cancer. Therefore, the present study was undertaken to study the antitumour activity of *Colocasia gigantea* in cultured HeLa cells.

MATERIALS AND METHODS

Chemicals

Doxorubicin was supplied by Getwell Pharmaceuticals, Gurgaon, India. Reduced glutathione (GSH), 1-chloro-2,4-dinitronbezene (CDNB), 5,5'dithio 2-nitrobenzoic acid (DTNB), ethylenediamine tetra-acetic acid (EDTA), bovine serum albumin (BSA), thiobarbituric acid (TBA), crystal violet were obtained from Sigma Chemical Co. (Bangalore, India).Trichloroacetic acid (TCA), hydrogen peroxide (H_2O_2)were procured from SD Fine Chemicals, Mumbai, India, whereas disodium hydrogen phosphate (Na_2HPO_4), Tris buffer (Tris (hydroxymethyl) aminomethane and ammonium oxalate were purchased from Merck India Limited, Mumbai, India. Trypsin EDTA 1X, MTT (3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide), MEM medium, fetal bovine serum (FBS), and reduced nicotinamide adenine dinucleotide (NADH) were requisitioned from HiMedia, Mumbai, India.

Collection of rhizomes and extraction

The matured and non-infected rhizomes of *Colocasia gigantea* (family- Araceae) were collected from Manipur. The plant was identified by PG Department of Botany, DM College, Imphal-West, Manipur, India and it was further authenticated by the Botanical Survey of India, Shillong, Meghalaya, India. The matured rhizomes were cleaned shade dried, their skinremoved and chopped into thin slices for easy and quick drying. The dried rhizomes were powered using an electrical grinder at room temperature. A known amount of powdered rhizome of *C. gigantea* was sequentially extracted in petroleum ether, chloroform, ethanol and distilled water in order of increasing polarity using a Soxhlet apparatus. Each extracts, except petroleum ether was concentrated in vacuo and stored at -70°C until further use. The ethanol extract was used for the entire study and henceforth it will be called as CGE.

Drug/s dissolution

Doxorubicin was freshly dissolved in MEM and the ethanol extract of *Colocasia gigantea* were freshly dissolved in distilled water and diluted and filter sterilized immediately before use.

Cell culture

HeLa and V79 cells were procuredfrom the National Centre for Cell Science, Pune, India. The cells were grown in 25 cm^2 culture flasks (Corning Inc., Corning, NY, USA) containing 5 ml Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum, 1% L-glutamine and 50 µg/ml gentamicin. The cells were incubated at 37°C in an atmosphere of 5% CO_2 in humidified air in a CO_2 incubator (Eppendorf AG, Hamburg, Germany).

Experimental Design

A fixed amount of cells were seeded into 96 well plates or culture flasks that were divided into the different groups depending on the experimental protocol:

MEM group: The cells of this group served as negative control group and did not receive any treatment.

CGE group: This group of cells was treated with different concentrations of CGE.

DOX group: The cell cultures of this group were treated with5, 10 and 20 µg/ml of doxorubicin (DOX) and served as positive control.

Determination of cytotoxicity by MTT assay

The cytotoxic effects of different concentrations of ethanol extract of *Colocasia gigantea* was studied by MTT assay in HeLa and V79cells as described by Mosmann (1983). Usually 10^4 cells were seeded into 96 well plates (HiMedia, Mumbai, India) in 100 µl minimum essential medium (MEM). The microplates were kept at 37ºC in a CO_2 incubator in an atmosphere of 5% CO_2 in 95 % humidified air and the cells were allowed to attach for 24 h. Next day different concentrations of CGE or doxorubicin were added into each well of the microplates and incubated in the CO_2 incubator. After 48 hours, MTT was added into each well and the microplates were incubated for another 2 hours. The drug containing media were removed and the insoluble purple formazan formed was dissolved using lysis buffer and incubated once again for 4 hours after which the absorbance was recorded at 560 nm using a microplate reader (Spectramax M2, Molecular Devices, Sunnyvale, CA, USA). The cytotoxicity was calculated using the formula: Control-Treatment/Control X 100.

Determination of optimum exposure time for cytotoxicity

A separate experiment was conducted to study the effect of treatment duration of CGE on the cytotoxicity, where grouping and other conditions were essentially similar to that described above except that the cells were exposed to CGE for 2, 4 and 6 h and the cytotoxicity was determined by MTT assay as described above.

Determination of anticancer activity

Another experiment was performed to evaluate the anticancer activity of CGE, where grouping and other conditions were similar to that described in the experimental design. The anticancer activity of CGE was determined by inoculating 10^6 exponentially growing HeLa cells into several culture flasks. The cells were allowed to attach for 24 h and were treated with 100, 200 and 300 µg/ml CGE.

After 2 hours of drug treatment the media were removed and the flasks were washed twice with sterile PBS, and dislodged by trypsin EDTA treatment and the Clonogenicity of cells was determined by clonogenic assay [11]. Usually 200 HeLa cells were seeded into several individual petridishes containing 5 ml MEM and left undisturbed for colony formation for another 11 days. After the end of day 11 the resultant colonies of cells were stained with 1 % crystal violet in methanol and scored. Plating efficiency (PE) of the cells was determined and surviving fraction (SF) calculated.

PE = (Number of colonies counted x 100) / (Number of cells seeded)

SF = (Number of colonies counted) / (Number of cells seeded) x (mean plating efficiency).

BIOCHEMICAL ASSAYS

A separate experiment was carried out to estimate the effect of 100, 200 and 300 µg/ml CGE on the activities of various antioxidants in HeLa cells at 2, 4 and 6h post drug treatment.The drug containing media were removed; the cells were washed with sterile PBS and displaced using trypsin EDTA treatment. The cells were pelleted and weighed and 5% homogenate of the cells was prepared in PBS (pH 7.4) using ultrasonicator (PCI Analytics Pvt. Ltd., Mumbai, India). The following assays were carried out:

Glutathione estimation

Glutathione estimation was carried out as described earlier [12]. Briefly, 1.8 ml of 0.2M Na_2HPO_4 was mixed with 40 µl 10 mM DTNB and 160 µl of cell homogenate. The mixture was allowed to stand for 2 minutes at room temperature and the absorbance was read against the blank at 412 nm in a UV-VIS Biospectrophotometer (Eppendorf India Limited, Kolkata, India).The blank consisted of distilled water instead of cell homogenate.

Glutathione - S – transferase estimation

Glutathione-s-transferase activity was estimated by the method of Habig *et al.,* (1974). Briefly, 0.5 ml of 0.1 M phosphate buffer pH 6.5, 0.1ml of 20mM CDNB, and 8.8 ml distilled water were incubated at 37°C for 10 min. After incubation, 0.5 ml of 20 mM GSH and 0.1 ml of cell homogenate were added. The absorbance was read at 340 nm at 1 min intervals for 6 minutes in UV-VIS Biospectrophotometer.

Catalase

The method of Aebi (1984) was followed for catalase estimation. Briefly, 20 µl of sample was

diluted with 1.98 ml of 50 mM phosphate buffer (pH 7.0) in a 3 ml cuvette and the reaction (maintained at 20ºC) was started by adding 1 ml of 30 mM H_2O_2. The decrease in absorbance was monitored at 240 nm for 60 seconds in a UV-VIS Biospectrophotometer.

Estimation of lipid peroxidation

Lipid peroxidation (LOO) assay was carried out by the method of Buege and Aust (1978). Briefly, 1 ml of cell homogenate was mixed with 2 ml of TCA-TBA-HCl reagent and mixed thoroughly. The reaction mixture was heated in a boiling water bath for 15 minutes, cooled immediately to room temperature, centrifuged at 1000 rpm for 10 min and supernatant was collected and its absorbance was read at 535 nm against blank in a UV-VIS Biospectrophotometer.

STATISTICAL ANALYSES

The statistical analyses were performed using Origin Pro 8. All the results are expressed as Mean ± Standard Error of the Mean (SEM). Experimental data were analyzed by one way ANOVA followed by Tukey's test for multiple comparisons for different parameters between the groups. A P value of < 0.05 was considered as significant. The experiments were repeated for confirmation and since the difference between the original and repeat experiments was statistically non-significant the data of both the experiments were combined and presented in tables and figures.

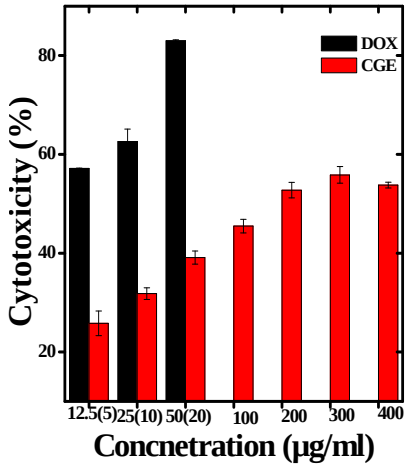

Figure 1: The cytotoxic effect of different concentrations of ethanol extract of *Colocasia gigantea* & doxorubicin on HeLa cells assessed by MTT assay. CGE- Ethanol extract of *Colocasia gigantea*, DOX- Doxorubicin. Figures in brackets indicate concentration of doxorubicin. The data represent Mean±SEM, N=5.

RESULTS

The results are expressed in fig. 1-9 as Mean ± Standard Error of the Mean (SEM).

Determination of Cytotoxicity

Treatment of Helga and V79 cells with different concentrations of CGE caused a concentration dependent increase in the cytotoxicity and the maximum cytotoxic effect was recorded for the highest concentrations of 300 µg/ml CGE. The cytotoxicity between of 200 and 300 µg/ml CGE was not statistically significant the former was chosen for other experiments (Figure 1). Similarly, CGE induced maximum cytotoxicity at 200 µg/ml in V79 cells (Figure 2). The positive control DOX also showed a similar pattern (Fig. 1-2).

Determination of treatment duration

The optimum CGE treatment duration for cytotoxic effect was also evaluated by MTT assay at 2, 4 and 6 hours. The CGE treatment resulted in a time dependent increase in the cytotoxicity in HeLa and V79 cells and maximum cytotoxic effect was observed in the cells treated with CGE for h respectively (Fig. 3-4). However, this increase was not statistically significant hence 2 h treatment duration was selected for further experiments.

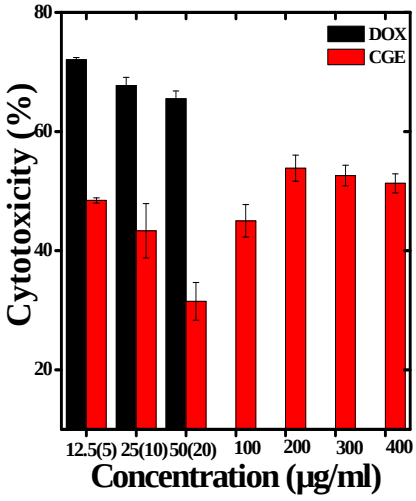

Figure 2: The cytotoxic effect of different concentrations of ethanol extract of *Colocasia gigantea* & doxorubicin on V79 cells assessed by MTT assay. CGE- Ethanol extract of *Colocasia*

gigantea, DOX- Doxorubicin. Figures in brackets indicate concentration of doxorubicin. The data represent Mean±SEM, N=5.

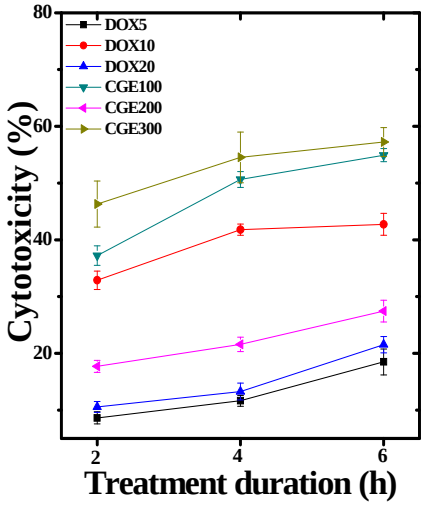

Figure 3: The effect of different concentration of the ethanol extract of *Colocasia gigantea* & doxorubicin on HeLa cells determined by MTT assay. Ethanol extract of *Colocasia gigantea* (CGE), Doxorubicin (DOX). The data represent Mean ± SEM, N=5.

Clonogenic Assay

Treatment of HeLa cells with different concentrations of CGE caused a concentration dependent decline in the clonogenicity of cells (Fig. 5). A maximum decline in the clonogenicity was observed for 300 µg/ml CGE, where the survivin g fraction of HeLa cell reached a nadir (0.22) less than half of 200 µg/ml (Fig. 5).

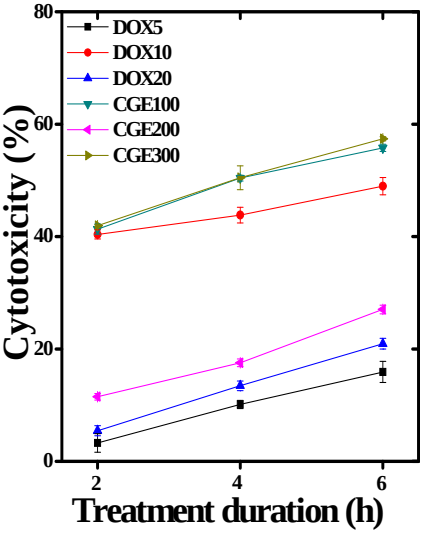

Figure 4: The effect of different concentration of the ethanol extract of *Colocasia gigantea* & Doxorubicin on V79 cells determined by MTT assay. Ethanol extract of *Colocasia gigantea* (CGE), Doxorubicin (DOX). The data represent Mean ± SEM, N=5.

g fraction of HeLa cell reached a nadir (0.22) less than half of 200 µg/ml (Fig. 5).

Glutathione

Treatment of HeLa cells with different concentrations of CGE caused a concentration dependent but significant depletion in glutathione contents at all the post-treatment times (Figure 6). The GSH concentration also declined in a time dependent manner and maximum decline was observed at 6 h post treatment (Fig. 5). The concentration of glutathione also declined in a similar as DOX treated group (Fig. 6).

Glutathione-s-transferases

GST actvity declined in a concentratoin depenent manner and it was significant lower than the MEM treated group. The acivit of GSt also reducted with time in the HeLa cells treated with diffrent oncentrations of CGE and a greatest decline was observed at 6 h post-treatment and for 300 µg/ml (Fig. 7).

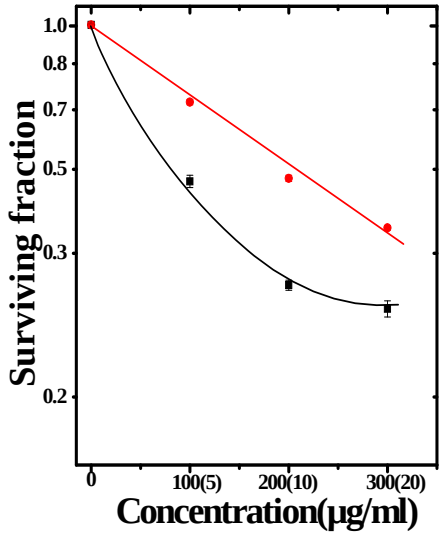

Figure 5: Effect of different concentrations of the ethanol extract of *Colocasia gigantea* & doxorubucin (DOX) treatment on the survival of HeLa cells. Figures in brackets on X-axis indicate concentration of doxorubicin. The results are expressed as Mean ± SEM. N=3. Squares: doxorubicin & Circles: ethanol extract of *Colocasia gigantea*

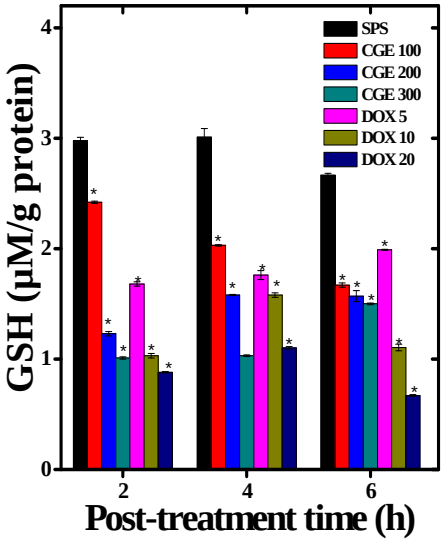

Figure 6: Alteration in the GSH activity of cultured HeLa cells treated with different concentrations of CGE and DOX. Minimum essential media (MEM), Ethanol extract of *Colocasia gigantea* (CGE) & doxorubicin (DOX). The data represent Mean±SEM, N=5. *p<0.01 when the treatment group are compared to MEM group. Standard error of the mean (SEM).

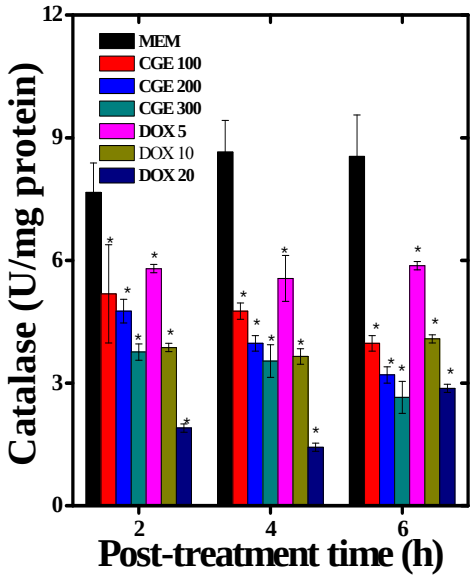

Figure 8: Alteration in the Catalase activity of cultured HeLa cells treated with different concentrations of CGE & DOX. Minimum essential media (MEM), Ethanol extract of *Colocasia gigantea* (CGE) & doxorubicin (DOX). The data represent Mean±SEM, N=5. *p<0.01 when the treatment group are compared to MEM group. Standard error of the mean (SEM).

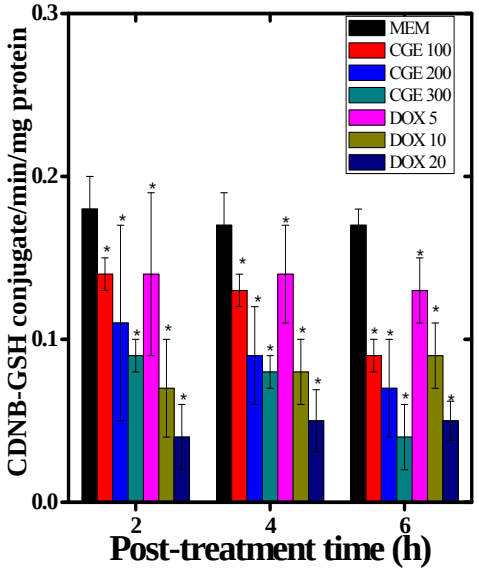

Figure 7: Alteration in the GST activity of cultured HeLa cells treated with different concentrations of CGE & DOX. Minimum essential media (MEM), Ethanol extract of *Colocasia gigantea* (CGE) & doxorubicin (DOX). The data represent Mean±SEM, N=5. *p<0.01 when the treatment group are compared to MEM group. Standard error of the mean (SEM).

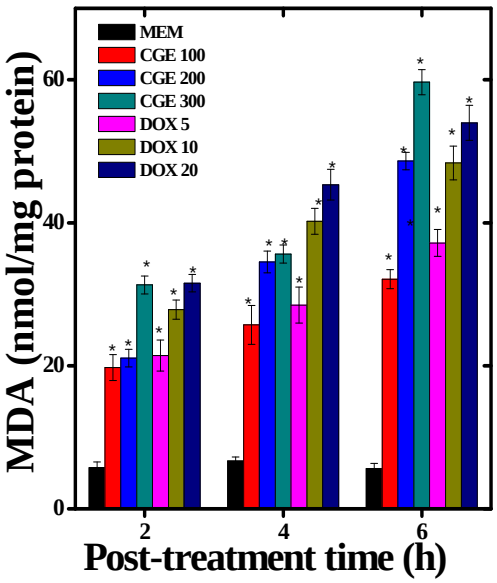

Figure 9: Alteration in the Lipid peroxidation activity of cultured HeLa cells treated with different concentrations of CGE & DOX. Minimum essential media (MEM), Ethanol extract of *Colocasia gigantea* (CGE) & doxorubicin (DOX). The data represent Mean±SEM, N=5. *p<0.01 when the treatment group are compared to MEM group. Standard error of the mean (SEM).

Catalase

The catalase activity also alleviated with increasing CGE concentration and there was significant reduction in the catalase activity at all post-treatment assay time when compared to MEM treatment. The analysis of catalase activity with time showed a time dependent decline in the catalase activity for all CGE concentrations and it was lowest at 6 h post treatment Fig. 8) The DOX treatment also resulted in an identical decline in catalase activity at all post treatment times (Figure 8).

Lipid Peroxidation

The treatment of HeLa cells with different concentrations of CGE induced LOO efficiently as indicated by a concentration dependent rise in the LOO at all post-treatment times (Fig. 9). This increase in LOO was significantly higher and it was at least 6 folds higher at 6 h post treatment in CGE treated group. The maximum LOO was detected at 6 h post treatment in all the groups (Fig. 9). The DOX treatment also showed a pattern similar to that of CGE treatment (Fig. 9).

DISCUSSION

The adverse effects induced by modern chemotherapeutic regimens and development of therapy resistance are the major stumbling block for successful treatment of tumors [13,-16]. The other disadvantage of systemic chemotherapy is induction of second malignancies due to genomic damge in the normal cells [17]. Therefore screening of newer paradigms that do not trigger the development of adverse effects and second malignancies are of crucial importance. The natural products and plants can provide the opportunity to develop non-toxic and effective drug molecules to treat cancer. Therefore the present study was undertaken to evaluate the anticancer potential of *Colocasia gigantea* in cultured HeLa cells.

The cytotoxic effect of any drug candidate/s can be ascertained by MTT assay, which is a rapid and standard technique to determine the cytotoxicity of any drug/treatment. The viable cells or metabolically active cells are able to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT into formazan by the action of mitochondrial succinate dehydrogenase enzyme and the level of activity is a measure of the viability of the cells. The weaker the color formed, the more are the dead cells. MTT assay has been used to test cytotoxicity of numerous drugs in various cell lines since its discovery [18]. The treatment of HeLa and V79 cells with ethanol extract of *Colocasia gigantea* caused a concentration dependent rise in the cytotoxicity. There are no reports regarding the evaluation of cytotoxicity of ethanol extract of *Colocasia gigantea.* However MTT assay has been used to investigate the cytotoxic effects of other plants in vitro [19-21]. The cytotoxic effect of CGE was further confirmed by clonogenic assay, whch is long term assay to study the toxicity of any agent. The CGE treatment led to a concentration dependent decline in the clonogenicity of *HeLa* cells. The cytotoxic effect of ethanol extract of *Colocasia gigantea*has not been studied yet. However the other medicinal plants like *Tinospora cordifolia*, and *Aphanmixis polystchya* and synthetic molecules including doxorubicin, daunorubicin and cytarabine have been reported to alleviate the clonognonic potential of cultured cells earlier [22-25]. Almost all cancer cells are at increased oxidative stress, which may be essential for progression and development of tumor.

The tumors also express high level of antioxidants to balance the increased oxidative stress and this increased antioxidant level is linked with the survival advantage in the tumor cells and also it helps to develop resistance to chemotherapy [26,27]. The excess oxidative stress induced by chemotherapeutic drugs is responsible for cell death as it stimulates various mechanism of cell death including non-apoptotic form of cell death [28]. Lipid peroxidation is a measure of oxidative stress as the products of lipid peroxidation damage the important macromolecules like proteins and nucleic acid which final lead to death of the cell [29,-31]. The CGE increased the oxidative stress in a concentration and time dependent manner and this may be the reason for effective cell killing in the present study. Most of the chemotherapeutic agents kill neoplastic cells by increasing oxidative stress in the tumor cells [32,33]. Malondialdehyde (MDA) is a major product of lipid peroxidation [34]. MDA has the ability to react with nucleic acid bases and form adducts to dG, dA, and dC [35]. Lipid peroxidation has been implicated in the pathogenesis of a number of diseases including cancer due to its ability to damage DNA and subsequent mutations in the tumor suppressor genes [30,36]. This property of

lipid peroxidation may be responsible for killing tumor cells in the present study. The glutathione is the most abundant non-protein intracellular antioxidant that has diverse role in numerous physiological processes [37].

The increase in glutathione has been implicated in tumor progression and resistance to chemotherapy and reduced glutathione levels have been reported to kill tumor cells more effectively [38-42]. A similar mechanism seems to operational in the present study where the treatment of HeLa cells with CGE has reduced the GSH concentration in a time and concentration dependent manner. The enzyme GST catalyzes the nucleophilic attack of glutathione (GSH) on electrophilic substrates by binding with glutathione on its hydrophilic G-site and its adjacent H-site with the electrophilic substrates to bring them in a close proximity. They also activate the sulfhydryl group on GSH, thereby allowing for nucleophilic attack of GSH on the electrophilic substrate [43].

Elevated levels of GST in tumor cells are associated with increased resistance to apoptosis [44,45]. The CGE reduced the GST activity in a concentration and time dependent fashion that may have induced effective killing of HeLa cells. Various GST inhibitors have been shown to modulate drug resistance by sensitizing tumor cells to anticancer drugs [46,47]. Catalase or oxidoreductase is present in all organisms and it detoxify H_2O_2 into water and oxygen and it is also involved in various other processes. High levels of catalase have been reported in patients with lung cancer, whereas decreased levels of catalase were indicated in breast cancer, head and neck cancer, gynaecological cancer, lymphoma, prostate cancer and urological cancer [48]. The over expression of catalase has been reported to reduce the apoptosis in tumor cells after chemotherapy [49]. The treatment of HeLa cells with CGE depleted the activity of catalase in concentration and time dependent manner, which would killed the HeLa cells effectively.

The mechanisms of cell killing by CGE are mostly not understood. However present study makes it very clear that CGE administration has increased the lipid peroxidation more than 6 fold thereby leading to a rise in the oxidative stress, which would have damaged the cellular DNA, other biomolecules and membranes killing the cells. The alleviated levels of GST, catalase and GSH would have further increased the oxidative stress and added insult to injury killing the HeLa cells effectively. The cancer and cancer cell lines over express the COX-II and nuclear transcription factors NF-κB and Nrf2 and they are also involved in resistance to tumor therapy [50-52]. The suppression of transcriptional activation of these genes by CGE may have played an important role in effectively killing the cells. The induction of apoptosis and activation of p53 and related proteins may have also contributed their share in bringing cell death.

CONCLUSIONS

The present study clearly demonstrates the cell killing ability of CGE and the cell killing may be due to the increased LOO, accompanied by a decline in the GSH, GST and catalase, that would have increased the oxidative stress that may have triggered the DNA, protein and membrane damage killing the cells effectively. CGE may have also suppressed the activation of COX-II, NF-κB and Nrf2 elements that may have induced apoptotic cell death. The over expression of p53 and related proteins may have also contributed to cell death in the present study.

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Research Article

FREE RADICAL SCAVENGING AND ANTIOXIDANT POTENTIAL OF DIFFERENT EXTRACTS OF *COLOCASIA GIGANTEA* (BLUME) HOOK. F. *IN VITRO*

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ABSTRACT

Free radicals have been implicated in various diseases including cancer. The agents that can inactivate the formation of free radicals or scavenge free radicals may be of great potential to reduce the oxidative stress induced health disorders in humans. The leaves and rhizomes of *Colocasia gigantea* form part of human diet as they are consumed by humans as vegetable regularly. The present study was undertaken to study the free radical scavenging activity of *Colocasia gigantea* in vitro. The rhizomes of *Colocasia gigantea* were collected, shade dried, powdered and sequentially extracted in chloroform, ethanol, and water. The antioxidant activity of various extracts was evaluated by their ability to inhibit the generation of DPPH, hydroxyl (˙OH), superoxide ($O_2^{\bullet-}$), $ABTS^{\bullet+}$and nitric oxide (NO˙) radicals in vitro. Total flavonoid and the total phenol contents were also determined to understand their role in free radical scavenging. The chloroform, ethanol, and aqueous extracts of *Colocasia gigantea* showed a concentration dependent inhibition in DPPH, ˙OH, $O_2^{\bullet-}$, $ABTS^{\bullet+}$ and NO˙ radical generation. Different extracts of *Colocasia gigantea* showed the presence of polyphenols. The *C. gigantea* scavenged DPPH, ˙OH, $O_2^{\bullet-}$, $ABTS^{\bullet+}$and NO˙ radicals in a concentration dependent manner and this activity may be due the presence of various polyphenols and flavonoids in its rhizomes.

Keywords: *Colocasia gigantea*, Free radical, polyphenols, flavonoids.

INTRODUCTION

A free radical is an atom or molecule with an unpaired electron in its outer most orbit[1], which is freely available for reaction. Such unpaired electrons make these species very unstable and highly reactive with other molecules and they try to pair their electron(s) and generate a more stable compound. The oxygen derived radicals also known as Reactive Oxygen Species (ROS) are an important class of radicals that are produced in living system for various purposes[2]. The ROS are dangerous species and are highly reactive with the molecules around them[3]. ROS is a collective term, which includes not only the oxygen radicals (Ö and OH) but also some non-radical derivatives of oxygen, including hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and ozone (O_3)[4].

The free radicals are usually produced in organisms that utilize oxygen for energy production, especially during oxidative phosphorylation in the mitochondria. Similarly, the reactive nitrogen species (RNS) are equally important in biological systems as they are involved in several cellular processes including cell signaling[5]. Antioxidant means "against oxidation." An antioxidant is any substance that retards or prevents deterioration, damage or destruction by oxidation[6]. Antioxidants includes several organic substances, including vitamin C, E, and A (which is converted from beta-carotene), selenium and a group known as carotenoids[7]. Despite, the fact that organisms have in built safety mechanisms to neutralize free radicals by different antioxidant molecules present in the cell, excess induction of ROS and RNS does occur. This excess generation of ROS and RNS may overwhelm the endogenous defense system and supplementation by exogenous antioxidants may be essential to neutralize the additional induction of ROS and RNS since the increased induction of ROS and RNS leads to several inflammatory diseases, especially autoimmune disorders, rheumatoid arthritis, cataract, aging, cardiovascular, gastrointestinal and neurodegenerative diseases and cancer[8]. The exogenous supply of antioxidants may be required to neutralize the deleterious effect of ROS/RNS and support the endogenous antioxidants system[9]. Currently available synthetic antioxidants like butylated hydroxyl anisole (BHA), butylated hydroxy toluene (BHT), tertiary butylated hydroquinone and gallic acid esters have been suspected to cause negative health effects, which has led to strong restrictions on their use in humans. This indicates that there is a need of non-toxic naturally occurring antioxidants, which do not trigger adverse effects. Recently, there has been an upsurge of interest in the therapeutic potentials of medicinal plants as antioxidants in reducing free radical-induced tissue injury. Many plant extracts and phytochemicals have shown to possess free radical scavenging activities[10] but generally there is still a need to find more information concerning the antioxidant potential of other plant species.

*Colocasia gigantea (*family: Araceae) also called giant elephant ear or Indian taro is 1.5-3 m tall herb with a whorl of large leaves at its apex. It bears a large, fibrous, and inedible corm. *C. gigantea* grows commonly in India, Thailand and other Southeast Asian countries[11]. The leaf stalks are used as a vegetable in some areas in, India, Southeast Asia and Japan. In India and Bangladesh, the tubers are cooked and used as a vegetable[12]. In Thailand, *C. gigantea* is considered as a minor food crop and is mainly utilized as a stem vegetable. *C. gigantea*'s stalks are often used for making homemade Thai food called "Bon curry". In the Pacific islands, the tubers are cooked and eaten as a starch[11]. In Thai traditional medicine, *C. gigantea* tuber is heated over a fire and is used to reduce

"internal heat" (fever) and also for the treatment of drowsiness. The fresh or dried tubers mixed with honey are used in the treatment of phlegm in northern Thailand. Fresh tubers have been shown to ameliorate stomach problems, combat infection, and accelerate the healing of wounds. Recently the leaf and tuber extracts have been found to be cytotoxic to HeLa cells[13]. The supplementation of antioxidants from dietary source will be of great importance than those given exogenously from other sources. Since not much information is available on *Colocasia gigantea* despite the fact that it is commonly used as a vegetable in India and Southeast Asia and is part of human diet, the present study was undertaken to investigate the antioxidant potential of different extracts of *Colocasia gigantea* in vitro.

MATERIALS AND METHODS

Collection and extraction of plant

The non-infected and matured rhizomes of *Colocasia gigantea* (family- Araceae) were collected from Manipur. The plant was identified by PG Department of Botany, DM College, Imphal-West, Manipur, India and further authenticated by Botanical Survey of India, Shillong, Meghalaya, India. The non-infected and matured rhizomes of *Colocasia gigantea* were collected and cleaned and chopped into small pieces. The rhizome pieces were spread into the stainless steel trays for drying under shade at room temperature in dark in clean and hygienic conditions to avoid entry of insects, animals, fungus, and extraneous terrestrial materials. The exhaust and free air circulation was allowed. The dried rhizomes were powdered in an electrical grinder at room temperature. Usually 100 g of rhizome powder was extracted sequentially in chloroform, ethanol and water in a Soxhlet apparatus, evaporated to dryness under reduced pressure and stored at -80°C until use. The chloroform, ethanol and water extracts of *Colocasia gigantea* will be called as CGC, CGE and CGA henceforth.

Chemicals

All the chemicals used were of analytical grade and Milli Q water was used for the entire analysis. 1,1- dimethylsulfoxide (DMSO), ascorbic acid, nitroblue tetrazolium (NBT), ethylenediaminetetra acetic acid (EDTA), trichloroacetic acid (TCA), sodium nitroprusside, and Griess reagent were procured from Sigma-Aldrich Chemical Co. Bangalore, India. Methanol, ethanol, sodium acetate, ferric chloride, Folin-Ciocalteau reagent, sodium carbonate, sodium hydroxide, sodium chloride, potassium chloride, disodium hydrogen phosphate (anhydrous), potassium dihydrogen phosphate, aluminum chloride, potassium acetate, gallic acid, glacial acetic acid and acetyl acetone were procured from Merck India, Mumbai.

Estimation of free radical scavenging in vitro

The scavenging activity of CGC, CGE and CGA was estimated using the following protocols:

DPPH free radical scavenging assay

The DPPH scavenging activity of CGC, CGE and CGA was carried out according to earlier described method[14] with minor modifications. To 20, 40, 60, 80, 100, 120, 140, 160, 180 and 200µg/ml CGC, CGE or CGA 1 ml of 0.1 mM DPPH in methanol was added. After thorough mixing, the mixture was kept in the dark for 30 min and the absorbance was measured at 523 nm using UV-VIS spectrophotometer (SW 3.5.1.0. Biospectrometer, Eppendorf India Ltd., Chennai). Methanol was utilized for the baseline correction. The results have been compared with that of the control prepared as above without sample. Radical scavenging activity has been expressed as a percentage and calculated using the following formula:

% Scavenging= (Acontrol-Asample)/ Acontrol X 100.

Where Asample is the absorbance of the test sample and Acontrol is the absorbance of the control.

Hydroxyl radical scavenging activity

Scavenging of the hydroxyl ($^{\bullet}OH$) free radical was determined by the earlier described method[15]. Briefly, the reaction mixture contained deoxyribose (2.8 mM), KH_2PO_4-NaOH buffer, pH 7.4 (0.05 M), $FeCl_3$ (0.1 mM), EDTA (0.1 mM), H_2O_2 (1 mM), ascorbate (0.1 mM) and 20, 40, 60, 80, 100, 120, 140, 160, 180,200,220 or 240 µg/ml CGC, CGE or CGA in a final volume of 2 ml. The reaction mixture was incubated for 30 min at ambient temperature followed by the addition of 2 ml trichloroacetic acid (2.8% w/v) and thiobarbituric acid. The reaction mixture was kept in a boiling water bath for 30 min, cooled and the absorbance was read at 532 nm in a UV-VIS spectrophotometer. The results have been expressed as gallic acid equivalent which was used as a standard.

Superoxide anion scavenging activity

Superoxide free radicals formed by alkaline DMSO react with NBT to produce coloured diformazan. Scavenging of the superoxide ($O_2^{\bullet-}$) anion radical was measured using a modified method[16]. Briefly, the reaction mixture contained 0.2 ml NBT (1 mg/ml in DMSO) and 0.6 ml of various concentrations (20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 µg/ml) of CGC, CGE or CGA , 2 ml of alkaline DMSO (1 ml DMSO containing, 5 m M NaOH in 0.1 ml water). The blank consisted of pure DMSO instead of alkaline DMSO. The absorbance was recorded at 560 nm in a UV/VIS spectrophotometer. The antioxidant capacity of the CGE based on its ability to inhibit formazan formation has been expressed as mg ascorbic acid equivalent /100 g of extract.

ABTS scavenging activity

ABTS scavenging activity of CGC, CGE or CGA was carried out as described earlier[17]. Briefly, 37.5 mg of potassium persulphate was dissolved in 1 ml of distilled water. 44 µl of this solution was added to 9.7 mg of ABTS dissolved in 2.5 ml of distilled water to prepare ABTS solution. The ABTS solution was allowed to stand in the dark at room temperature for 12-16 hours. The working solution consisted 1 ml of ABTS solution, 88 ml of 50% ethanol. 25 µl of different concentrations (20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 µg/ml) of the different extracts of *C. gigantea* were mixed with 250 µl of the working ABTS solution and allowed to react for 4 minutes. The absorbance was then measured at 734 nm in a UV-VIS spectrophotometer. Gallic acid was used as the standard antioxidant and the activity was expressed as gallic acid equivalent. The percentage scavenging activity was calculated as follows:

Scavenging (%) = Control O.D – Sample O.D/ Control O.D x 100.

Nitric oxide scavenging activity

The nitric oxide scavenging activity was estimated by spectrophotometric method[18]. Briefly, sodium nitroprusside (5 mM) in phosphate buffer saline was mixed with 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220 and 240 µg/ml CGC, CGE or CGA and incubated at 25°C for 150 min. The samples were mixed with Greiss reagent (1% sulfanilamide, 2% H_3PO_4, and 0.1% napthylenediamine dihydrochloride). The absorbance of the chromophore formed during diazotization of nitrite with sulfanilamide and subsequent coupling with napthylenediamine was read at 546 nm using a UV-VIS spectrophotometer. The inhibition of nitric oxide formation was determined with respect to standard potassium nitrite treated in the same way with Greiss

reagent. The results have been expressed as potassium nitrite equivalent which has been used as a standard.

Determination of Total phenol contents

The total phenol contents were estimated by Folin-Ciocalteau reagent[19], where 20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 µg/ml CGC, CGE or CGA after dilution or gallic acid (standard phenolic compound) were mixed with Folin-Ciocalteau reagent (5 ml, 1:10 diluted with distilled water) and aqueous Na_2CO_3 (4 ml, 1M). The mixture was allowed to stand for 15 minutes and the total phenol contents were measured at 756 nm with a UV-VIS spectrophotometer. The total phenol contents are expressed in terms of gallic acid equivalent (mg/100 g of extracts).

Total Flavonoids Determination

The total flavonoid contents in CGC, CGE or CGA were estimated using Aluminum chloride method as described earlier[20]. Different concentrations of *C. gigantea* extract(20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 µg/ml) were separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1M potassium acetate and 2.8 ml distilled water and incubated at room temperature for 30 min. The absorbance was recorded at 415 nm with a UV-VIS spectrophotometer. Quercetin was used as the standard and the results have been expressed as quercetin equivalent.

RESULTS

The results are shown in table 1-4 and figures 1-7.

DPPH radical scavenging activity

Various extracts of *Colocasia gigantea* showed a concentration dependent rise in the scavenging of DPPH radicals as indicated by the discolouration of DPPH which is purple in colour (Table1). Maximum scavenging was observed at a concentration of 140µg/ml for CGC, CGE or CGA that ranged between 50.41±0.30, 51.08±0.68 to 51.2±0.23 mg of ascorbic acid equivalent, respectively (Figure 1).

Hydroxyl Radical Scavenging activity

Different extracts of *C. gigantea* inhibited the generation of hydroxyl radical in a concentration dependent manner and a maximum inhibition in OH generation was observed at 200µg/ml for chloroform, ethanol and aqueous extracts, respectively (Figure 2). When the efficiency of scavenging was determined with respect to gallic acid equivalent the most effective extract was CGA followed by, CGE and CGC, respectively (Table 2).

Superoxide anion scavenging activity

The chloroform, ethanol and aqueous extracts of *C. gigantea* showed a concentration dependent rise in the inhibition of superoxide radical generation up to a concentration of 140µg/ml that declined thereafter (Figure 3). The maximum effect was observed for ethanol extract which scavenged superoxide radical more efficiently than the other two extracts and this was 59.17±0.23 mg ascorbic acid equivalent, whereas it was 51.02±0.07mg and 50.63±0.11 mg ascorbic acid equivalent for chloroform and aqueous extracts, respectively (Table 1).

ABTS scavenging activity

The different extracts of *C. gigantea* showed a concentration dependent rise in the scavenging activity of the ABTS free radicals up to a concentration of 140µg/ml CGE and CGC and declined thereafter, whereas a maximum scavenging effect for aqueous extract was recorded at 180 µg/ml and a decline thereafter (Figure 4 and Table 2).

Nitric oxide scavenging activity

Various extracts of *C. gigantea* showed a concentration dependent increase in the scavenging activity of nitric oxide radicals and a highest scavenging of NO was observed for 140µg/ml for chloroform and ethanol, whereas and for aqueous extracts showed the highest scavenging activity at 120µg/ml (Figure 5). Among all the three extract the best effect was observed for CGA that revealed maximum activity at a lower concentration than the other two extract (Figure 5 and Table 3).

Total phenol contents

The presence of phenolic compounds in the CGC, CGE and CGA was estimated as total phenol contents that increased in a concentration dependent manner up to 200µg/ml for chloroform, ethanol and aqueous extracts, respectively (Figure 6). The CGA showed the least amount of total phenols when compared to CGE and CGC, where the amount was maximum (Table 4).

Total flavonoid contents

The total flavonoid contents in CGC, CGE and CGA increased in a concentration dependent manner up to 200µg/ml equivalent to gallic acid contents (Table 4). The maximum amount of total flavonoid was estimated for CGC followed by CGA, whereas it was least for CGE (Table 4).

Table 1: DPPH and superoxide radical scavenging activities of different extracts of *Colocasia gigantea*.

Conc. (µg/ml)	DPPH (%±SEM)			Superoxide (%±SEM)		
	Aqueous	Ethanol	Chloroform	Aqueous	Ethanol	Chloroform
20	26.75±0.38	36±0.14	32.33±0.24	26.06±0.11	11.16±0.45	25.61±0.13
40	30.83±1.04	41±0.14	34.6± 0.11	28.86±0.08	14.29±0.34	28.13±0.07
60	34.25±0.38	44±0.38	37.13±0.17	29.22±3.24	26.20±0.60	31.26±0.04
80	39.08±0.22	48.5±0.14	39.46±0.24	37.89±0.37	31.46±0.64	33.89±0.04
100	41.25±0.62	48.91±0.60	41.33±0.40	43.76±0.31	38.88±0.87	37.31±0.04
120	43.91±0.36	54.08±0.68	46.6±0.23	49.45±0.30	52.15±0.19	47.86±0.10
140	50.41±0.30	51.33±0.65	51.2±0.23	50.63±0.11	59.17±0.23	51.02±0.07
160	45.91±0.44	49.91±0.22	46.86±0.35	47.53±0.11	56.87±0.19	48.92±0.09
180	44.41±0.22	47.83±0.50	42.8±0.11	45.87±0.08	54.27±0.16	47.83±0.16
200	42.91±0.20	45.91±0.22	40.2±0.07	43.85±0.11	52.33±0.20	46±0.05

Data are Mean± SEM, N=5.

Table 2: Hydroxyl and ABTS scavenging activities of different extracts of *Colocasia gigantea*.

Conc. (µg/ml)	Hydroxyl Radical (%±SEM)			ABTS (%±SEM)		
	Aqueous	Ethanol	Chloroform	Aqueous	Ethanol	Chloroform
20	22.16±0.15	22.87±0.13	24.05±0.15	23.87±0.19	26.12±0.33	24.35±0.62
40	23.76±0.18	23.75±0.13	26.33±0.10	25.35±0.07	32.96±0.12	26.79±0.11
60	26.51±0.28	30.22±0.15	30.22±0.26	28.11±0.05	35.43±0.09	30.70±0.08
80	32.47±0.37	33.90±0.13	34.44±0.18	30.02±0.15	37.85±0.29	38.34±0.05
100	40.32±1.28	36.77±0.07	35.42±0.18	33.42±0.30	41.61±0.22	38.50±0.22
120	38.31±0.23	41.31±0.10	36.89±0.23	36.90±0.05	43.18±0.19	45.70±0.17
140	48.38±0.39	44.75±0.18	39.07±0.10	42.73±0.45	46.88±0.04	48.17±0.13
160	49.86±0.56	46.66±0.10	42.87±0.43	49.34±0.10	50.28±0.52	51.47±0.14
180	57.83±0.24	51.07±0.28	46.25±0.91	53.75±0.07	47.39±0.09	45.98±3.25
200	60.94±0.36	56.39±0.13	51.91±0.10	49.37±0.22	44.64±0.07	42.15±2.90
220	54.31±0.54	56.39±0.13	46.97±0.10	-	-	-
240	48.32±0.34	48.50±0.18	42.30±0.53	-	-	-

Data are Mean± SEM, N=5.

Table 3: Effect of different extracts of *Colocasia gigantean* on nitric oxide scavenging.

Conc. (µg/ml)	Nitric Oxide Radical (%±SEM)		
	Aqueous	Ethanol	Chloroform
20	19.59±0.77	22.09±0.67	25.19±0.45
40	22.80±0.50	26.74±0.67	27.82±0.69
60	34.50±0.77	28.68±1.02	31.49±0.45
80	42.10±0.50	32.94±1.02	38.58±0.45
100	46.49±0.50	39.92±1.02	40.94±0.45
120	54.97±0.77	45.34±0.67	48.03±0.45
140	50.87±0.50	53.10±1.02	54.85±0.69
160	49.12±0.50	47.28±1.02	47.50±0.94
180	42.69±0.77	33.72±0.67	43.56±0.69
200	35.08±1.01	28.68±1.02	30.97±0.69
220	32.49±0.77	26.74±0.67	27.56±0.45
240	30.69±0.50	23.68±1.02	24.85±0.69

Data are Mean± SEM, N=5.

Table 4: Total phenol and total flavonoid contents of different extracts of *Colocasia gigantea.*

Conc. (µg/ml)	Total Phenols (µg/ml ±SEM)			Total Flavonoids (µg/ml ±SEM)		
	Aqueous	Ethanol	Chloroform	Aqueous	Ethanol	Chloroform
20	84.16±2.20	102.50±1.44	114±1.73	271.15±8.96	189.06±20.36	280.60±15.79
40	90.00±1.44	162.5±12.82	184±2.64	353.83±19.74	213.93±4.97	331.35±5.17
60	113.33±7.12	204.16±2.20	258±1.73	385.58±15.13	231.35±4.30	373.15±10.76
80	147.50±1.44	240.00±1.44	264±1.73	447.78±4.30	253.74±4.30	420.91±10.34
100	162.50±1.44	289.16±2.20	310±3.60	502.50±8.96	308.47±6.58	459.72±7.89
120	161.66±4.63	333.33±2.20	365±2.64	537.33±4.30	350.76±8.61	501.51±5.17
140	182.50±1.44	355.00±1.44	424±2.64	567.18±4.30	393.05±8.96	588.08±16.62
160	205.00±1.44	387.50±1.44	488±2.64	634.35±12.92	432.85±4.30	680.62±5.17
180	222.5±1.44	430.83±3.63	527±2.64	684.10±6.58	457.73±6.58	761.22±15.51
200	265±1.44	505.83±2.20	576±1.73	726.39±6.5	849.53±6.58	829.88±7.89

Data are Mean± SEM, N=5

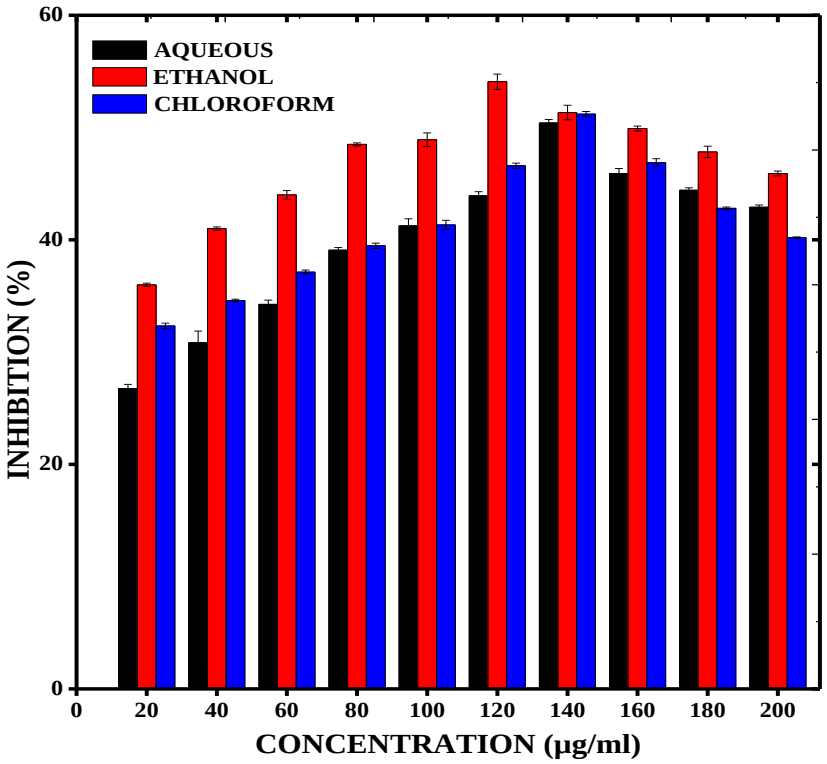

Figure 1: Effect of different extracts of *Colocasia gigantean* on DPPH radical scavenging activity. Data are Mean± SEM, N=5

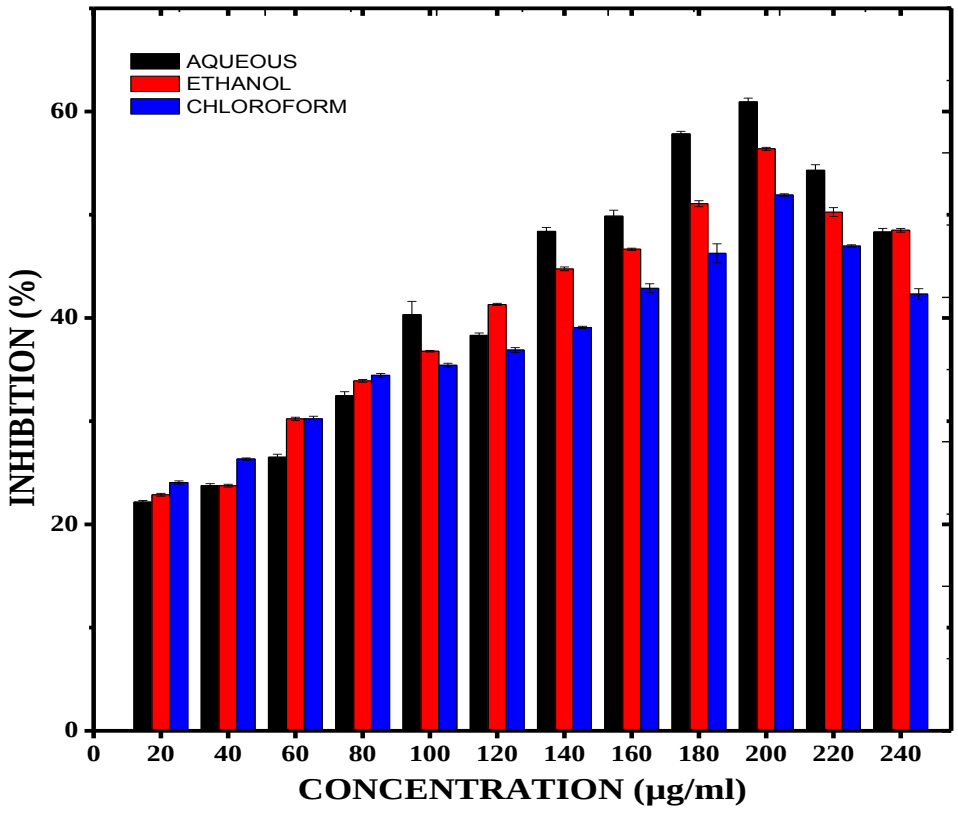

Figure 2: Hydroxyl radical scavenging activity of different extracts of *Colocasia gigantea* expressed as gallic acid equivalent. Data are Mean± SEM, N=5

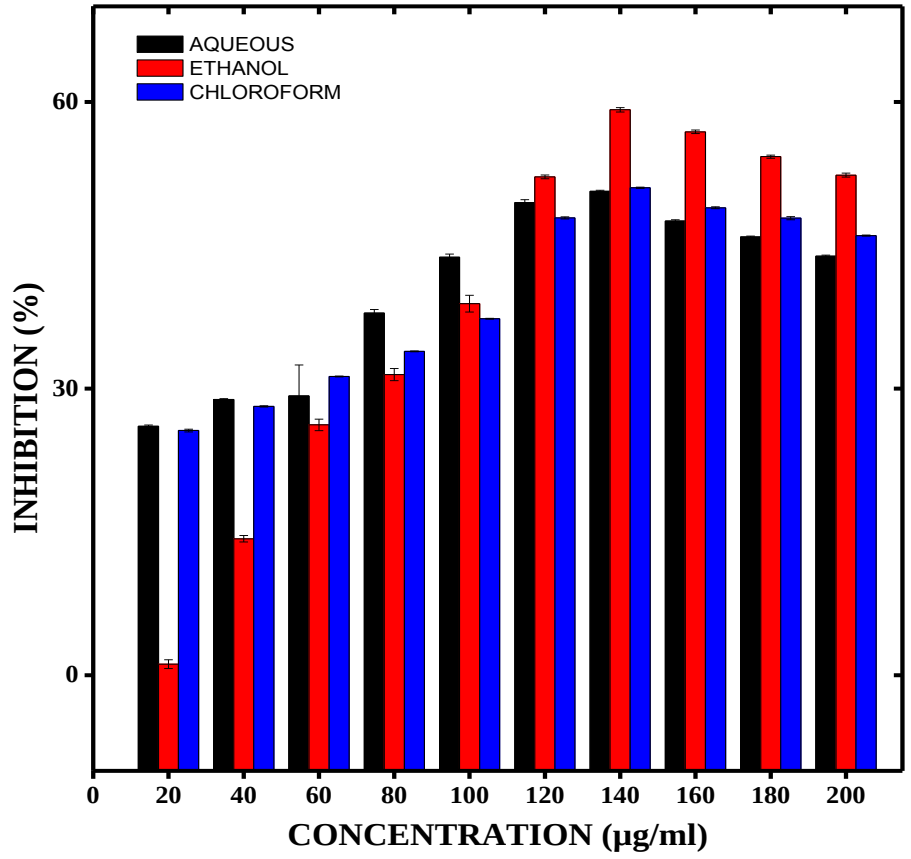

Figure 3: Superoxide radical scavenging activity of different extracts of *Colocasia gigantea*. Data are Mean± SEM, N=5.

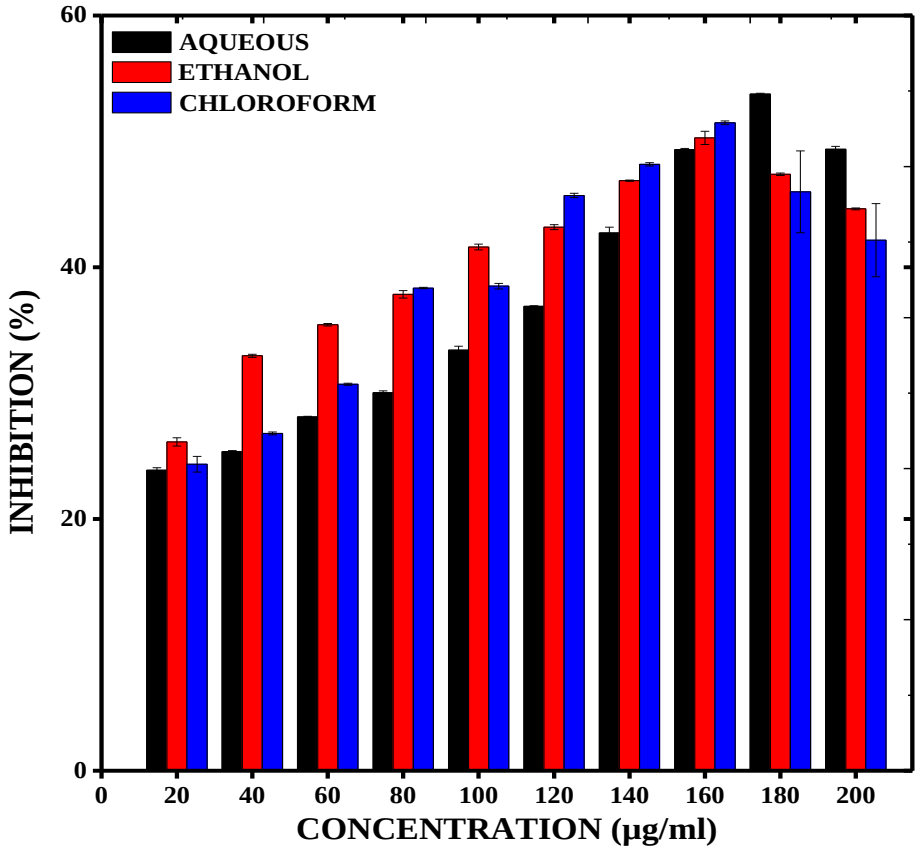

Figure 4: ABTS radical scavenging activity of different extracts of *Colocasia gigantea*. Data are Mean± SEM, N=5.

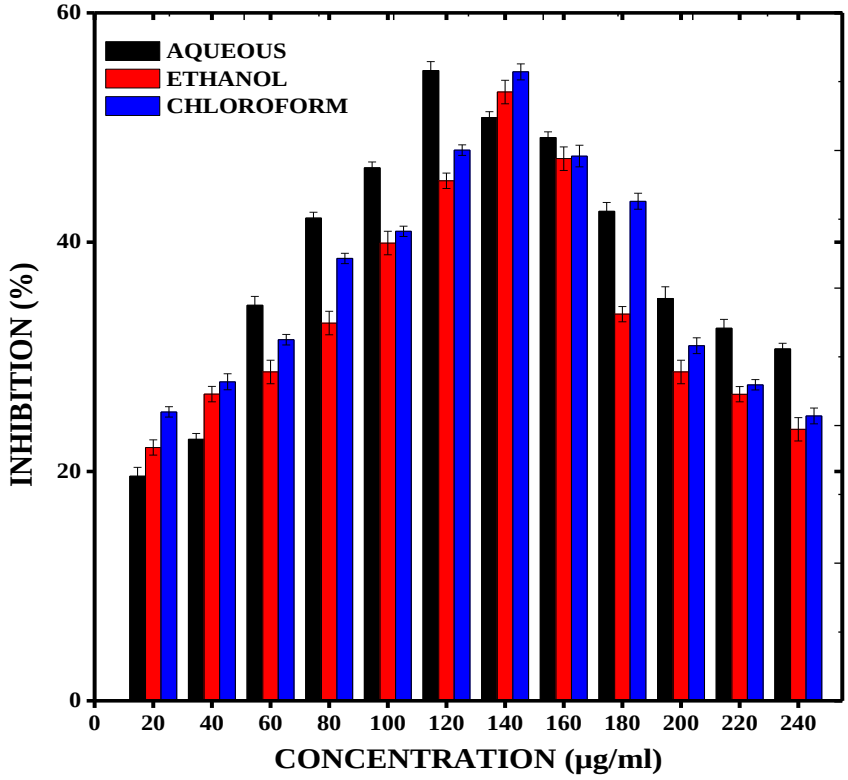

Figure 5: NO radical scavenging activity of different extracts of *Colocasia gigantea* (. Data are Mean± SEM, N=5

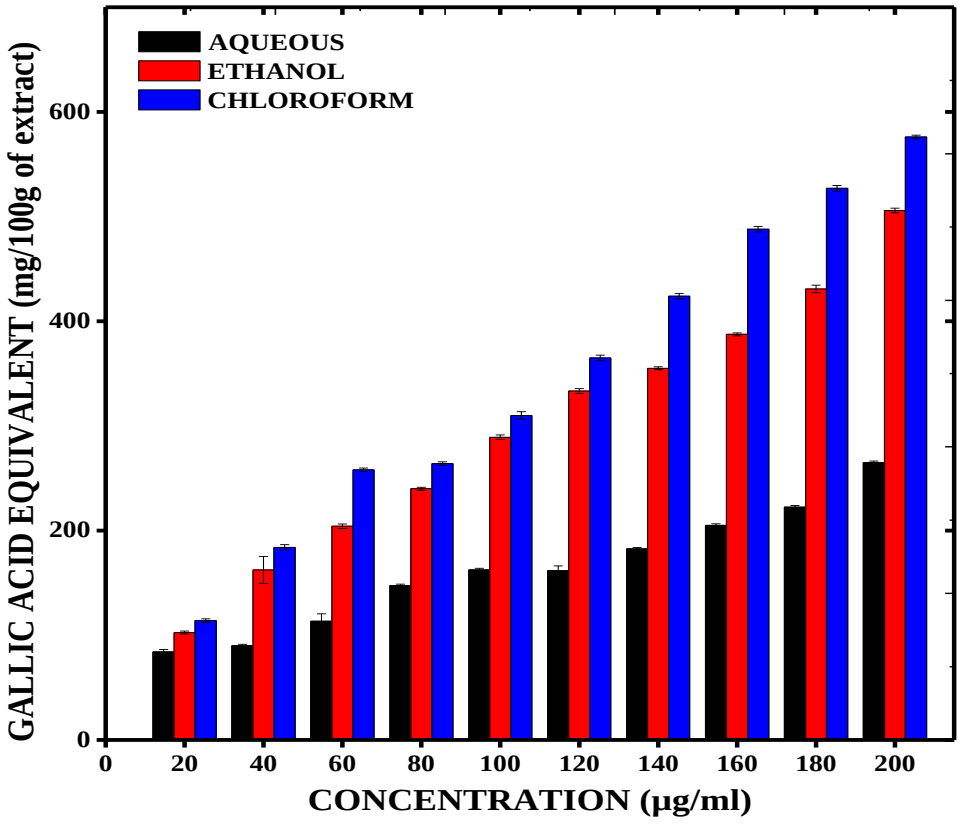

Figure 6: Total phenol contents of different extracts of *Colocasia gigantea* (20-200µg/ml). Data are Mean± SEM, N=5.

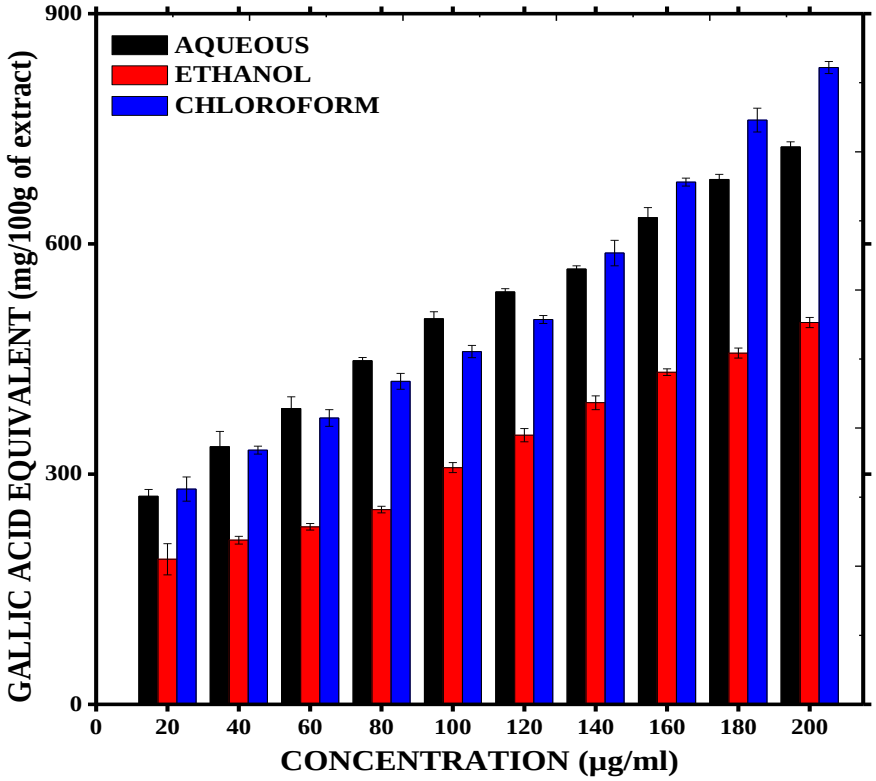

Figure 7: Total flavonoids contents of different extracts of *Colocasia gigantea* (20-200µg/ml). Data are Mean± SEM, N=5.

DISCUSSION

The oxidative stress is the price organisms have to pay for using oxygen as a chemical energy source that is required for various activities. The oxidative stress is induced due to the production of free radicals during various metabolic activities and respiration in particular. The cells are equipped with a repertoire of antioxidant or antioxidant enzymes that usually take care of the normal oxidative stress induced during respiration however in situation of excess oxidative stress it may not be possible for the endogenous antioxidant system to passivate it. Moreover, generation of excess oxidative stress has been indicated as a causative factor of several disorders including, aging, autoimmune, benign oral, cardiovascular, kidney, liver, intestine, and Alzheimer diseases and arthritis, diabetes and cancer [21]. It is also known that external supplementation with antioxidants have been helpful in reducing the risk of oxidative stress. It will be better if the antioxidants come from the dietary sources. The *Colocasia gigantea* or Indian taro is part of human diet and it is consumed frequently during the season when it is available. Therefore, the present study was undertaken to investigate the antioxidant activity of various extracts of *Colocasia gigantea* in vitro.

DPPH (1, 1-diphenyl-2-picrylhydrazyl) assay is one of the simple and convenient techniques to estimate the antioxidant property of any agent. DPPH is a stable free radical by virtue of the delocalization of the free electron over the molecule, which is violet coloured, and faints into the yellow coloured congener (DPPH-H) once it accepts an electron donated by the antioxidant and subsequently get reduced with a high λ-shift in the visible spectra (from 520 nm to 330 nm). This redox process was first reported by Goldschmidt and Renn (1922). All extracts of *Colocasia gigantea* scavenged DPPH free radical in a concentration dependent manner up to 140µg/ml. Somewhat similar results have been reported for *Agele marmelos*, *Croton caudatus*, *Milletia pachycarpa*, *Schima wallichi*, *Eleagnus caudata*,*Castanopsis indica* and *Dysoxylum gobara*, *Oroxylum indicium*[10,21,22]. The other phytochemicals including naringin, and mangiferin have been reported to scavenge DPPH free radicals in a concentration dependent manner[23].

The hydroxyl free radical is highly reactive species, which reacts in the close vicinity of its formation[24]. During respiration superoxide radical is converted into H_2O_2 which is a highly toxic and oxidizing agent. Despite H_2O_2 is not very reactive, it becomes highly toxic to the cell due to its ability to generate hydroxyl radical in the cells in presence of metals by Haber Weiss and/or Fenton reaction[15]. The hydroxyl radical is capable of inducing detrimental effects on the important macromolecules including proteins and nucleic acids. It reacts with DNA leading to base and sugar damages[24,25]. Hence, neutralization of hydroxyl radical is crucial to protect cells from its deleterious effects. The various extracts of *Colocasia gigantea* inhibited the generation of OH radicals in a concentration dependent manner and hence it may be a useful agent to inactivate this radical in vivo. Many other plant extracts and flavonoids have been found to scavenge hydroxyl free radicals in a concentration dependent manner[21-23,26,27].

The superoxide free radical is an intermediate during cellular respiration which is produced as a result of incomplete metabolism of oxygen[28]. It has been reported to play important role in cell signaling[29]. However, the superoxide anion produces H_2O_2, which in turn generates hydroxyl free radicals in the presence of metals leading to pathologic alteration of several important macromolecules in the cell[30]. Therefore, neutralization of superoxide radical will be able to arrest the chain of ROS generation and protect the cells from the oxidative stress. The various extracts of *C. gigantea* have been found to inhibit the production of superoxide radical in a concentration dependent manner. Other plant extracts and some flavonoids

have been reported to scavenge the superoxide anion free radical earlier[21-23,26,27].

The nitric oxide radical (NO•) is a labile molecule and it is generated in mammalian cells as a byproduct of respiration. It is also used by neutrophils to eliminate invading bacteria. NO• also plays an important role in signal transduction and nerve conduction. However, excess production of NO• is toxic, especially after reaction with oxygen or superoxide anion radicals and the reaction products includes NOx and ONOO- (peroxynitrite). These products are able to inflict severe cellular damage[31]. The various extracts of *C. gigantea* reduced the generation of NO• in a concentration dependent manner. Several plant extracts and plant formulations have also been reported to scavenge NO• in a concentration dependent manner[28]. Similarly, some of the plant flavonoids including naringin and mangiferin have been reported to scavenge nitric oxide free radical in a concentration dependent manner earlier[21-23,26,27,32].

The $ABTS^{\bullet+}$ chromophore was produced through the reaction between ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) and potassium persulfate which is indicated by blue/green colouration. The addition of antioxidants to the pre-formed radical cation reduces the ABTS, indicated by the discoloration of the blue/green colour. The extent of decolorization was expressed as the percentage inhibition of the $ABTS^{\bullet+}$[17]. This trapping of ABTS derived radical cation ($ABTS^{\bullet+}$) by free radical scavengers is a commonly employed method to evaluate the total charge of antioxidants present in complex mixtures[33]. The inhibitory action of $ABTS^{\bullet+}$ indicates the antioxidant potential of any chemical agent. The different extracts of *C. gigantea* did inhibit the generation of $ABTS^{\bullet+}$ radical in a dose dependent manner.

A similar effect has been observed with *Agele marmelos*, *Syzygium cumini* earlier[22, 31]. The exact mechanism of free radical scavenging by *C. gigantea* is not known. However, the free radical scavenging and antioxidant activity of *C .gigantea* may be due to the presence of various phytochemicals like polyphenols and flavonoids, which may be able to donate or accept electron thus neutralizing their oxidative effects. Plants produce phenolic compounds and flavonoids in particular as secondary metabolites that help plants in pollination, to ward off against fungal attacks and also give attractive colours to flowers[34]. These flavonoids have been reported to exert a conducive effect on human health owing to their free radical scavenging ability and antioxidant nature.

CONCLUSION

The present study indicates that all the extracts of *C. gigantea* showed a concentration dependent inhibition of free radicals. These activities of *C. gigantea* may be due to the presence of various phenolic compounds and flavonoids. Therefore, *C. gigantea* could be a potential source of natural antioxidant which may act as therapeutic agent in preventing or slowing down the progression of oxidative stress related degenerative diseases.

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Antitumor activity of different extracts of *Colocasia gigantea* in Dalton's lymphoma transplanted Swiss Albino mice

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Abstract

The acute toxicity was determined in normal non-tumour bearing mice administered with different doses of various extracts of *Colocasia gigantea* orally or and intraperitoneally. The oral administration of chloroform, ethanol and aqueous extracts of *Colocasia gigantea* were non-toxic up to 2 g/kg body weight. The intraperitoneal administration of different extracts of *Colocasia gigantea* exhibited toxic effect and the LD50 for ethanol extract was found to be 0.2 g/kg b. wt., whereas it was 0.15 g/kg b. wt. for chloroform and aqueous extracts, respectively. The determination of anticancer activity by intraperitoneal administration of 0, 50, 75, 100, 125, 150, 175, 200 or 225 mg/kg b. wt. of ethanol extract into Dalton's lymphoma transplanted mice resulted in a dose dependent rise in tumour regression and increase in the average survival as well as median survival time. The maximum tumour free survivors were observed at 200 mg/kg b. wt. of ethanol extract and further experiments were carried out using this dose. The evaluation of micronuclei showed that the ethanol extract *Colocasia gigantea* increased the frequency of micronucleated mononucleate cells as well as micronucleated binucleate cells in a time dependent manner and their frequencies were maximum at 36 h post-treatment. Similarly, *Colocasia gigantea* ethanol extract increased the apoptosis index in a time dependent manner and the highest apoptosis was observed at 36 h post treatment. The biochemical studies revealed a significant decline in the glutathione concentration, glutathione-s-transferase, superoxide dismutase and catalase activities accompanied by elevated lipid peroxidation. The safety of ethanol extract of 200 mg *Colocasia gigantea* was ascertained by evaluating aspartic acid transaminase, and alanine aminotransferase, creatinine and uric acid at different post treatment times in the liver and kidney of Dalton's lymphoma bearing mice. These parameters did not show any significant alteration and they were within the normal range. The ethanol extract of *Colocasia gigantea* did not show any toxicity orally however, intraperitoneal administration did exert toxic effects and it also induced anticancer activity in tumour cells by increasing tumour free survivors. The cytotoxic effect of ethanol extract may be due to induction of DNA damage in the form of micronuclei and apoptosis and reduction in glutathione, glutathione-s-transferase, superoxide dismutase and catalase accompanied by a rise in the lipid peroxidation.

Key words: *Colocasia gigantea*, Dalton's lymphoma, micronculei, glutathione and apoptosis

INTRODUCTION

The cardiac diseases are the leading cause of death and cancer is the second largest killer disease globally. Despite development of various modern treatment modalities, the mortality rates, especially for solid tumors remains a major cause of concern. It is also projected that mortality rates due to cancer may out number cardiovascular diseases not in too distant a future in the developed world [1]. The chemotherapy is one of the important modalities to treat cancer and the term "chemotherapy" was introduced by Paul Ehrlich in the early nineteen century who defined chemotherapy as the use of chemicals to treat diseases. However, it became associated with cancer treatment in 1943 since then treatment of cancer with chemicals is synonymous with chemotherapy [2]. Several synthetic chemicals find their use in the cancer treatment and role of natural products in cancer treatment was realized when alkaloids isolated from *Catharanthus roseus* were found to be useful in the treatment of hematologic malignancies [3]. Thereafter several other molecules have been isolated from different plants to treat different types of cancers [4]. Podophyllotoxins separated from the ethanol extract of *Podophyllum peltatum* also showed anticancer activity against a wide range of tumors. The etoposide and teniposide, which are derivatives of podophyllotoxins are in frequent clinical use [5]. Camptothecins are natural product derived cancer chemotherapeutic agents that have found their application in clinics to treat different cancers [6]. The taxols are another class of natural products isolated from plants and are in frequent clinical use to treat a wide range of neoplastic disorders [7].

The chemical synthesis of natural products further strengthened their use and may still continue to play a significant role in the treatment of cancer in the years to come [2,8]. The currently used chemotherapy for cancer treatment has several side effects and therefore there is a need for better therapy with lesser side effects [9]. Besides, the high cost as well as lack of effectiveness of the current conventional therapies (chemotherapy and radiation), especially for solid tumors, use of plants for cancer treatment may be alternative medical strategy to treat cancer [10]. The side effects due to most cancer drug/s induced toxicity also act as a driving force to the use of alternative medicine for better cure [11]. Plants are not only safe for long term therapy but also provide nutrition and reduce the side effects of conventional cancer therapy. The high cost and negative impact of conventional therapy, low-cost and safety of plants has been drawing increased attention towards plants and plant derived products for cancer cure [12]. Plants and natural products are still in great demand due to their safety, efficacy and lesser side effects [13] and about 80% individuals in the developing countries still depend on plants to treat different diseases. There are also reports that 25% of modern drugs are obtained from plants and 70% of the drugs introduced in the United States for the past 25 years have their origin in plants [4,14]. Plants contain many phytochemicals which work in a synergistic mode of action in such a way that their uses can complement or damage others or neutralize their possible negative effects. The use of multicompounds is preferred over the use of single drug for the treatment of several diseases including cancer, AIDS, diabetes, etc. due to their beneficial effects [15]. The popularity of use

of herbal medicines by general public is due to the belief that botanicals will provide some measure of benefit over and above modern allopathic medical approaches. They are also considered non-toxic or less toxic than the synthetic molecules.

*Colocasia gigantea (*family: Araceae) commonly called giant taro or elephant's ear, is a large, stemless, tuberous, frost-tender perennial herb, which typically grows up to 4-7' tall and has wide and heart-shaped to arrowhead-shaped, conspicuously-veined, downward-pointing, peltate, dull green to gray green leaves (2-4' long) attached to stout, succulent stems. As the common name suggests, each leaf purportedly resembles the ear of an elephant. It is native to valley forests in China and Southeast Asia. In Fiji, the locals make use of either boiled or baked breadfruit or tubers of taro as slices along with roasted pig [16]. Along with culinary items of taro it is used as medicine to treat constipation and tuberculosis in Hawaii [17]. Pressed juice of petiole of taro is highly cystic and is even said to arrest arterial hemorrhage [18]. Taro is used as medicine in China [19]. Nutritionally, taro is very similar to tannia. It contains starch 17.5% amylose and the rest as amylopectin. Starch grain is very small and the size ranges from 1-4 μm. It is rich in most of the essential amino acids and hence is considered to be a good leafy vegetable. It is reported that 100 g of taro tuber contains 73.1 g moisture, 3 g protein, 0.1 g fat, 1.7 g minerals, 22.1 g carbohydrates, 0.04 g calcium, 0.14 g phosphorus, 2.1 mg iron, 80 IU Vitamin B and trace of Vitamin C [20]. Since not much information is available on *Colocasia gigantea* despite the fact that it is commonly used as a vegetable in India and Southeast Asia as a part of human diet, the present study was undertaken to study the anticancer activity of *Colocasia gigantea* in mice transplanted with Dalton's lymphoma cells.

MATERIALS AND METHODS

Chemicals

Dimethylsulphoxide (DMSO), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), nitrobluetetrazolium (NBT), phenazinemethosulphate (PMS), reduced glutathione (GSH), triton X-100, ethylenediaminetetra-acetic acid (EDTA), sodium pyruvate, thiobarbituric acid (TBA), ethidium bromide, acridine orange, crystal violet, and cytochalasin B were obtained from Sigma Aldrich Chemical Co. (Kolkata, India). Sodium carbonate (Na_2CO_3), trichloroacetic acid (TCA), potassium chloride (KCl), potassium sodium-tartrate, and hydrogen peroxide (H_2O_2), were procured from SD Fine-Chemicals Ltd., Mumbai, India, whereas sodium hydroxide (NaOH), sodium chloride (NaCl), disodium biphosphate (Na_2HPO_4), sulphuric acid (H_2SO_4), hydrochloric acid (HCl), n-butanol, Tris (hydroxymethyl) aminomethane (Tris buffer) and ammonium oxalate were supplied by Merck India Limited, Mumbai. Phenol-chloroform-isoamyl alcohol (PCI), and reduced nicotinamide adenine dinucleotide (NADH) were purchased from HiMedia, Mumbai, India. Doxorubicin, Getwell Pharmaceuticals, Gurgaon, India.

Collection and Preparation of the Extract

The non-infected and matured rhizomes of *Colocasia gigantea* (family- Araceae) were collected from Manipur. The plant was identified by PG Department of Botany, DM college,

Imphal-West, Manipur, India and further authenticated by Botanical Survey of India, Shillong, Meghalaya, India. The non-infected and matured rhizomes were cleaned chopped into thin slices to facilitate drying in shade at room temperature. The dried rhizomes were powdered in an electrical grinder at room temperature. A sample of 100 g of powder was extracted sequentially with chloroform, ethanol and water in a Soxhlet apparatus [21]. The extract was then concentrated to dryness under reduced pressure and stored at -80 until further use. The chloroform, ethanol and water extracts of *Colocasia gigantea* will be called as CGC, CGE and CGA henceforth.

Preparation of Drug and mode of administration

The chloroform (CGC) ethanol (CGE) and aqueous (CGC) extracts of *Colocasia gigantea* were dissolved in appropriate solvent immediately before use, Doxorubicin was dissolved in sterile physiological saline (SPS). Each animal from each group received different treatments according to body weight intraperitoneally.

Animal care handling

The animal care and handling were carried out according to the guidelines issued by the World Health Organization, Geneva, Switzerland and the INSA (Indian National Science Academy, New Delhi, India). Swiss albino mice were bred before use in a controlled environment of temperature (24-25ºC), 50% humidity and light and dark (12 h each) cycle. Usually 5-6 animals were housed in a sterile polypropylene cage containing paddy husk (procured locally) as bedding material. Six to eight weeks old male and female Swiss albino mice weighing 28-35 g were procured from the inbred colony for experimentation. The animals were fed with commercially available food pellets and water ad libitum. The Institutional Animal Ethics Committee of Mizoram University, approved the entire study vide letter no. MZUIAEC16-17-01, Aizawl, India.

Acute toxicity determination

The acute toxicity of all extracts was determined both orally and intraperitoneally according to guidelines issued by the Organization for Economic Co-operation and Development (OECD). Albino mice selected by random sampling technique (n=10) of both sexes (5 males and 5 females) were used for each dose of individual extract. The animals were fasted for 18 hours (both food and water were withdrawn) prior to oral or intraperitoneal administration of different extracts of *Colocasia gigantea*. The control group received sterile physiological saline (SPS). The animals were weighed before and after fasting to estimate their weight loss. If mortality was observed in 2-3 animals then the dose administered was assigned as toxic dose. If mortality was observed in one animal, then the same dose was repeated again to confirm the toxicity. The LD_{50} for each extracts was calculated using Probit analysis.

Tumor Model

Dalton's lymphoma ascites (DLA) tumor, procured from the Department of Zoology, North-Eastern Hills University, Shillong was propagated in 10-12 weeks old mice by serial

intraperitoneal transplantation of 1 x 10^6 viable tumor cells in 0.25 ml PBS, pH 7.4 under aseptic conditions.

Experimental design

Dalton's lymphoma tumor bearing mice were divided into the following groups:

Negative Control groups. The negative control group were administered SPS alone.

DOX groups: This group of animals was injected with 0.5 mg/kg body weight of doxorubicin, a standard anticancer drug and served as positive control.

CGE groups: This group of animals received 50, 75, 100, 125, 150, 175, 200 or 225 mg/kg body weight of the ethanol extract of *Colocasia gigantea.*

The tumor bearing animals were given treatment once daily 1 day after tumorization for subsequent 9 days [22]. Each group consisted of ten animals for each dose. The animal survival was monitored daily up to 120 days, since the survival of animals up to 120 days is equivalent to 5 years survival in humans [23]. The deaths, if any, of the tumor bearing mice were recorded daily and the survival was determined. The tumor response was assessed by calculating median survival time (MST) and average survival time (AST). The MST and AST were calculated from the animals dying within 120 days and those surviving beyond 120 days were excluded from the study [22]. The increase in median life span (% IMLS), increase in average life span (% IALS) and the increase in life span (%ILS) was also calculated using the formulae:

MST= First death + Last death in the group/2

AST= Sum of animals dead on different days/No. of animals

IMLS (%) = MST of treated mice – MST of control x 100/MST of control

IALS (%) = AST of treated mice – AST of control x 100/AST of control

ILS (%) = (T/C x 100) - 100

Where, T is the mean survival days of treated mice and C is that of the control mice.

Micronucleus Assay

A separate experiment was performed to study the ability of ethanol extract of *Colocasia gigantea* to induce DNA damage in DLA cells .The grouping and other conditions were similar to that described in the experimental design section, except that the animals were injected with 200 mg/kg b. wt. CGE and the micronuclei were assayed at 12, 24 and 36 h post CGE treatment. The micronuclei were prepared according to the modified method of Fenech and Morley, (1985). Briefly, the DLA cells were aspirated from tumor bearing mice one hour after the last administration of SPS or CGE. The cells were washed twice with sterile PBS and 1X10^6 cells were inoculated into each well of 6 well sterile plates containing 3 ml of MEM and were

allowed to attach for 6 h. Thereafter the cells were treated with 3 µg/ml of cytochalasin-B to block cytokinesis. The cells were left undisturbed and allowed to grow for different times and terminated at 12, 24 and 36 h after the initiation of the cultures. The media containing cytochalasin-B were removed, the cells were washed twice with PBS, dislodged by trypsin EDTA treatment and pelleted by centrifugation at 1000 rpm for 5 min. The cell pellet was disturbed and cells were kept in mild hypotonic (0.75% ammonium oxalate) at 37°C, centrifuged again and the resultant cell pellet was fixed in Carnoy's fixative 3:1 (Methanol:Acetic acid). The cells were pelleted again by centrifugation, and resuspended in a small volume of fixative. The cells were spread on to pre cleaned coded slides so as to avoid observer's bias. The slides containing cells were stained with 0.25% acridine orange (BDH, England, Gurr Cat. No. 34001 9704640E) in Sorensen's buffer (pH 6.8) and subsequently washed in the buffer to remove excess stain. The Sorensen's buffer mounted slides were observed under a DM-2500 fluorescence microscope (Leica Microsystems, Wetzlar, Germany) equipped with 450–490 nm BP filter set with an excitation at 453 nm using a 40 X N Plan objective. A minimum of one thousand binucleate cells with well-preserved cytoplasm was scored for each culture and usually five cultures were used for each group. A minimum of 5000 cells were scored for each culture for the determination of the frequency of micronucleated binucleate cells (MNBNC). The micronucleated cells were scored according to the criteria of Kirsch-Volders *et al.,* (2003) and Fenech *et al.,* (2003).

Apoptosis Assay

A separate experiment was conducted to determine whether *Colocasia gigantea* has the ability to enhance apoptosis in Dalton's lymphoma cells. The grouping and other conditions were essential similar to that described for micronucleus assay. DLA cells were aspirated from tumor bearing mice one hour after the last administration of SPS or CGE. The cells were washed twice with sterile PBS and $1X10^6$ cells were inoculated into each well of 6 well sterile plates containing 3 ml of MEM. The cells were allowed to grow for 12, 24 and 36 h to assess apoptosis. The induction of apoptosis was studied at 12, 14 and 36 h post drug treatment as described earlier (Ribble et al., 2005). The tumor cells were aspirated and washed with ammonium chloride to lyse the erythrocytes and cells were pelleted by centrifugation. The cells were washed again with sterile PBS and spread on to clean coded slides and stained with freshly prepared ethidium bromide and acridine orange (1:1) stain (Sigma Aldrich Chemical Co. Bangalore, India) and observed under a DM-2500 fluorescent microscope (Leica Microsystems, Wetzlar, Germany). The number of live, and apoptotic cells were counted. A total of 1000 cells were counted for each slide and a total of 5000 cells were counted for each group. The viable cells were recognized by green fluorescent nuclei with organized structure, whereas the early apoptotic cells showed highly condensed or fragmented yellow chromatin in the nuclei. The cells showing orange chromatin, highly condensed and fragmented nuclei were considered as late apoptotic cells. The apoptotic cells also exhibited membrane blebbing as one of the morphological features. Only cells with yellow, condensed, or fragmented nuclei

were counted as apoptotic cells in a blinded, non-biased manner. The apoptotic index was calculated as follows:

Apoptotic index (%) = Number of apoptotic cells scored X 100/Total number of cells counted.

Biochemical Assays

The alteration in biochemical profile after administration with 200 mg/kg b.wt. CGE was assayed by conducting a separate experiment where grouping and other conditions were essentially similar to that described for apoptosis assay. The animals were sacrificed after nine days of drug treatment at an interval of 2, 4, 6, 8, 12 and 24 hours. Both the treated and untreated Dalton's lymphoma cells were aspirated under sterile conditions, washed with ammonium chloride followed by sterile phosphate buffer saline and pelleted. The cell pellets were weighed and 10% homogenate was prepared in cold sterile PBS (pH 7.4) and used for the estimation of various antioxidant and lipid peroxidation.

Estimation of Glutathione

Glutathione contents were estimated as described earlier [24]. In brief, 1.8 ml of 0.2 M Na_2HPO_4 was mixed with 40 µl of 10 mM DTNB and 160 µl of cell homogenate and allowed to stand for 2 minutes. The absorbance was read against the blank at 412 nm in a UV-VIS Biospectrophotometer (Eppendorf India Limited, Kolkata, India). The blank consisted of distilled water instead of cell homogenate.

Estimation of Glutathione - S – Transferase

Glutathione-s-transferase was determined by the method of Habig *et al.,* (1987). Usually, 0.5 ml of 0.1 M phosphate buffer (pH 6.5), 0.1 ml of 20mM CDNB, and 8.8 ml distilled water were incubated at 37°C for 10 min followed by the addition of 0.5 ml of 20 mM GSH and 0.1 ml of cell homogenate. The absorbance was read at 340 nm with a UV-VIS spectrophotometer at 1 min intervals for 6 minutes. Distilled water was used as a blank.

Catalase Assay

Catalase was assayed according to technique described by Aebi (1984). The 20 µl of cell homogenates was mixed with 1.98 ml of 50 mM phosphate buffer (pH 7.0) in a 3 ml cuvette. The reaction (maintained at 20ºC) was started by adding 1 ml of 30 mM H_2O_2. The decrease in absorbance was monitored at 240 nm for 60 seconds.

Superoxide Dismutase Assay

The activity of SOD was estimated as described by Fried (1975). 100 µl of cell homogenate, 100 µl of 186 µM phenazenemethosulfate, 300 µl of 3.0 mM nitrobluetetrazolium, and 200 µl of 780 µM NADH were incubated for 90 seconds at 30°C. The reaction was terminated by the addition of 1000 µl of acetic acid and then 4 ml n-butanol. The absorbance was read at 560 nm using UV/VIS spectrophotometer. The percent inhibition was calculated by measuring the absorbance of blank without SOD enzyme. The SOD activity was calculated using the formula (Blank-Sample)/Blank X 100.

Lipid Peroxidation Assay

Malondialdehyde (MDA) formed from the breakdown of polyunsaturated fatty acids, serve as a convenient index for determining the extent of peroxidation reaction of lipids. MDA has been identified as the product of lipid peroxidation (LOO) that reacts with thiobarbituric acid to give a red species absorbing at 535 nm. LOO assay was carried out following the method of Buege and Aust, 1978. One ml of cell homogenate was added to 2 ml of TCA-TBA-HCl reagent and was mixed thoroughly and heated in a boiling water bath for 15 minutes, cooled immediately at room temperature and centrifuged at 1000 rpm for 10 min. The supernatant was collected and its absorbance was read at 535 nm against blank in a UV-VIS spectrophotometer. The blank contained all the reagents except the cell homogenate substituted with distilled water. The MDA concentration of the sample was calculated using the extinction coefficient of $1.56x10^{6}M^{-1}cm^{-1}$.

Determination of Liver and Kidney function

An experiment was conducted to study the toxic effect of CGE where grouping and other conditions were similar to that of biochemical assay. A 10% homogenate of liver and kidneys was prepared in PBS using a homogenizer (Remi, India). The aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were estimated in the liver, whereas uric acid and creatinine were measured in the kidney homogenates with the help of commercial available Respons kits using a Respons 910 autoanalyzer (Diagnostic Systems GmbH¸ Holzheim, Germany).

Statistical Analyses

The statistical analyses were done using Origin Pro 8 SRO v8.0724 (B724), Northampton, MA, USA. The significance for survival analysis was determined by Kaplan Meier test and student's 't' test was applied for biochemical studies followed by Tukey's post -hoc tests for multiple comparisons, wherever necessary. The Wilcoxson's signed rank test was utilized for micronucleus and apoptosis assays. The results were confirmed by repetition of the experiments. Test of homogeneity was applied to determine any statistical differences between the repeat experiments. Since no significant differences were observed the data of all experiments were combined and expressed as mean ± standard error of the mean (SEM).

RESULTS

The results are expressed as the mean ± standard error of the mean (SEM), wherever required in Tables 1-16 and Figures 1-11.

Acute toxicity

Oral administration of the different extracts of *Colocasia gigantea* showed no signs of toxicity up to 2 g/kg b. wt. The acute toxicity assay after the intraperitoneal administration was carried out by up and down method. Based on the animal survival, chloroform and aqueous extracts showed an LD_{50} of 625 mg/kg b. wt. and 710 mg/kg b. wt. respectively, whereas ethanol extract was less toxic as the LD_{50} was823mg/kg b. wt. (Table 4-5).

Table 1: The survival of mice administered intraperitoneally with different doses of chloroform extract of *Colocasia gigantea* rhizomes.

Dose (g/kg b. wt.)	Mortality (%) 0n different days														Total	Remarks
	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
3	100	-	-	-	-	-	-	-	-	-	-	-	-	-	0	Aggressive, dull and all died within first day.
2.5	-	-	30	-	-	20	-	-	-	20	-	-	-	-	30	Aggressive, dull and 70% died before 14 days.
2	-	30	-	-	-	-	10	-	-	-	20	-	-	-	40	Aggressive, dull and 60% died before 14 days.
1.5	-	-	20	-	-	-	20	-	-	10	-	-	-	-	50	Dull, lethargic and 50 % died before 14 days.
1	-	20	-	-	-	10	-	-	-	10	-	-	-	-	60	Dull, lethargic and 60 % died before 14 days.
0.5	-	-	-	10	-	-	-	-	-	-	-	10	-	-	80	Dull and 20 % died before 14 days.
0.2	-	-	-	10	-	-	-	-	-	-	-	-	-	-	90	Active and 10% died before 7 days.
0.15	-	-	-	-	-	-	10	-	-	-	-	-	-	-	90	Active and 10% died on 7 days.
0.1	-	-	-	-	-	-	-	-	-	-	10	-	-	-	90	Active and 10% died before 14 days.
0.05	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100	Active and all survived.

N=10 for each dose

Table 2: The survival of mice administered intraperitoneally with different doses of ethanol extract of *Colocasia gigantea* rhizomes.

Dose (g/kg b. wt.)	Mortality (%) on different days														Total	Remarks
	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
3	10	-	-	-	-	-	-	-	-	-	-	-	-	-	0	Aggressive, Dull and died within first day.
2.5	-	30	-	20	-	-	20	-	-	-	20	-	-	-	30	Aggressive, dull and 70% died before 14 days.
2	-	-	20	-	-	-	-	-	20	10	-	-	-	-	50	Aggressive, dull and 50% died before 14 days.
1	-	-	10	-	-	-	-	-	20	-	-	-	-	-	60	Dull, lethargic and died before 14 days.
0.5	-	-	-	-	-	-	-	-	-	10	-	-	-	-	80	Dull, lethargic and 40% died before 14 days.
0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	90	Dull and died before 7 days.
0.15	-	-	-	-	10	-	-	10	-	-	-	-	-	-	90	Dull and died before 10 days.
0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	90	Active and 10% died before 14 days.
0.05	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100	Active and all survived.

N=10 for each dose

Table 3: The survival of mice administered intraperitoneally with different doses of aqueous extract of *Colocasia gigantea* rhizomes.

Dose (g/kg b. wt.)	Mortality (%) 0n different days														Total	Remarks
	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
3	100	-	-	-	-	-	-	-	-	-	-	-	-	-	0	Aggressive, dull and all died within 3 hrs.
2.5	100	-	-	-	-	-	-	-	-	-	-	-	-	-	0	Aggressive, dull and all died within first day.
2	20	-	-	-	30	-	-	-	10	-	-	-	10	-	30	Aggressive, dull and 70% died before 14 days.
1.5	-	20	-	-	-	-	10	-	-	-	-	20	-	-	50	Dull, lethargic and 50 % died before 14 days.
1	-	10	-	-	-	-	20	-	-	10	-	-	-	-	60	Dull, lethargic and 60 % died before 14 days.
0.5	-	-	-	-	20	-	-	-	-	-	-	-	-	-	80	Dull and 20 % died before 7 days.
0.2	-	-	-	10	-	-	-	-	-	-	-	-	-	-	90	Active and 10% died before 7 days.
0.15	-	-	-	-	-	-	10	-	-	-	-	-	-	-	90	Active and 10% died on 7 days.
0.1	-	-	-	-	-	-	-	-	-	10	-	-	-	-	90	Active and 10% died before 14 days.
0.05	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100	Active and all survived.

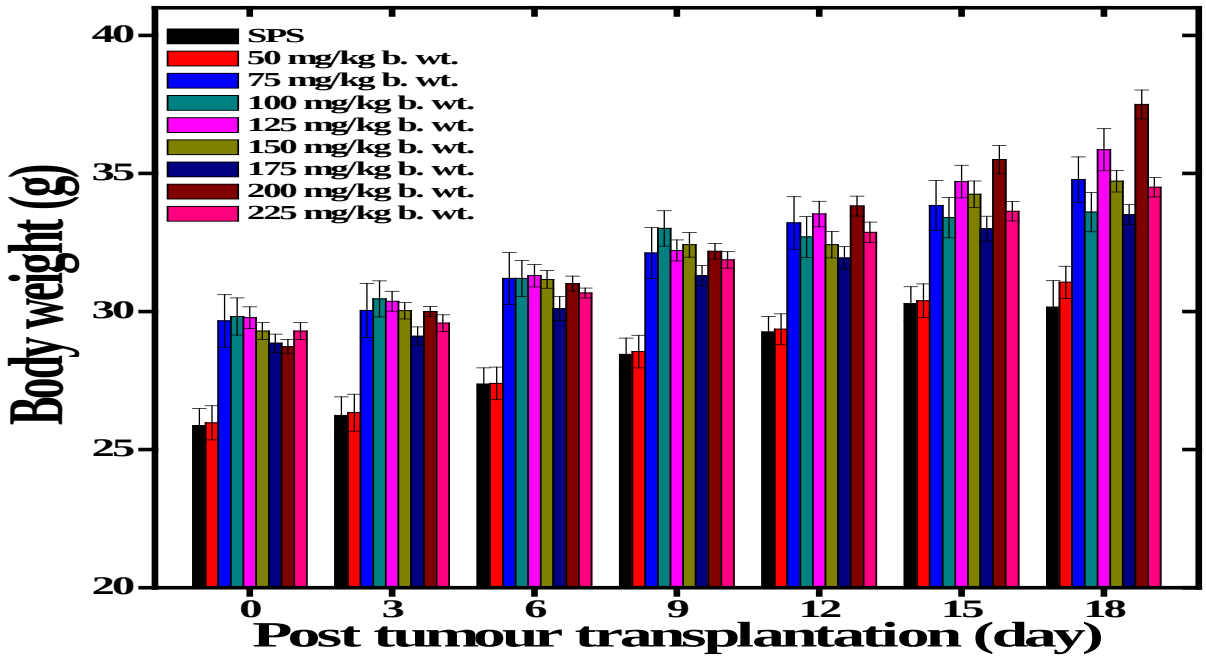

Figure 1: Body weight change of Dalton's lymphoma ascites bearing Swiss Albino mice by treating with ethanol extract of *Colocasia gigantea*. N=10.

Table 4: Acute toxicity of different solvent extracts of *Colocasia gigantea* administered orally in Albino mice.

Extract/ Group	Sex	Dose (g/kg.bwt.)	Body weight (g)			Survival
			Before fasting	After fasting	Loss (18 h)	
Control (SPS)	M	0	30	27	3	> 14 days
			32	29.8	2.2	> 14 days
			28.2	25	3.2	> 14 days
	F		30	28.9	1.1	> 14 days
			25.8	23.2	2.6	> 14 days
			27	24	3	> 14 days
Chloroform	M	2	29.4	27.5	1.9	> 14 days
			33	30	3	> 14 days
			29.3	27.4	1.9	> 14 days
			31	29.6	1.4	> 14 days
			29.6	27.5	2.1	> 14 days
	F		27	24	3	> 14 days
			29.6	26.3	1.3	> 14 days
			29	27	2	> 14 days
			26.2	25	1.9	> 14 days
			27.6	25.7	1.8	> 14 days
Ethanol	M	2	34.2	31.3	2.9	> 14 days
			32	30.6	1.4	> 14 days
			29.7	28.6	1.1	> 14 days
			27.5	25.8	1.7	> 14 days
			28.7	27.5	1.2	> 14 days
	F		33.1	29	4.1	> 14 days
			30.7	28.7	2	> 14 days
			32.4	30.4	2	> 14 days
			35.4	31	4.4	> 14 days
			32.8	30.8	2	> 14 days
Aqueous	M	2	35.3	33.2	2.1	> 14 days
			33.6	30.6	3	> 14 days
			34.5	32	2.5	> 14 days
			29.7	28.3	1.4	> 14 days
			28.8	26.4	2.4	> 14 days
	F		30.5	28.7	1.8	> 14 days
			31.6	28.8	2.8	> 14 days
			33.6	31.7	1.9	> 14 days
			28.7	28.9	1.8	> 14 days
			29.6	27.7	1.9	> 14 days

N=10 for each dose

Body weight changes

The mice transplanted with DLA cells gained weight continuously due to proliferation of tumor cells until the animal succumbed to death. The tumorized mice did not exhibit signs of tumour regression in the negative control group. The treatment of DLA mice with 50, 75, 100, 125, 150, 175, 200 or 225mg/kg body weight of ethanol extracts of *Colocasia gigantea* exhibited slight elevation in the body weight (Figure 1). The comparison of *Colocasia gigantea*

extract treated groups with negative control revealed a significant reduction in the body weight due to decrease in the cell proliferation (Table 6).

Table 5: Determination of acute toxicity in Swiss albino mice administered various doses of different extracts of *Colocasia gigantea* intraperitoneally.

Extract type	Dose (mg/kg b. wt.)	Survival (%)	LD_{50} (mg/kg)
Chloroform	50	100	625
	100	90	
	150	90	
	200	90	
	500	80	
	1000	60	
	1500	50	
	2000	40	
	2500	30	
	3000	0	
Ethanol	50	100	823
	100	90	
	150	90	
	200	90	
	500	80	
	1000	60	
	2000	50	
	2500	30	
	3000	0	
Aqueous	50	100	710
	100	90	
	150	90	
	200	90	
	500	80	
	1000	60	
	1500	50	
	2000	30	
	2500	0	
	3000	0	

The LD_{50} is determined using Probit analysis. N=10 for each dose

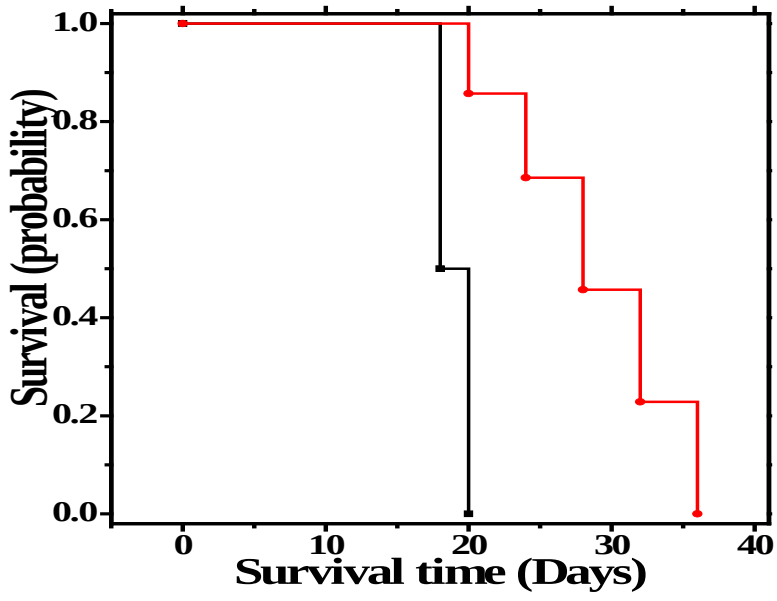

Figure 2(a): The survival of Dalton's lymphoma ascites bearing mice treated for 9 days consecutively with SPS or doxorubicin. Squares: SPS (Control); Circles: Doxorubicin (DOX). N= 10.

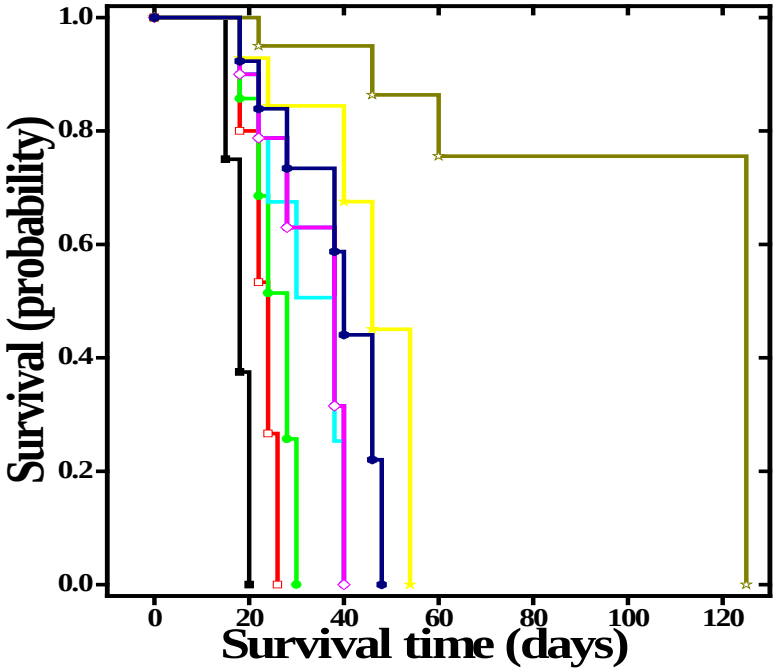

Figure 2(b): Kaplan Meirs' estimate of survival of Dalton's lymphoma ascites bearing mice treated for 9 days consecutively with different doses of ethanol extract of *Colocasia gigantea*. Closed squares: SPS; Open squares: 50 mg/kg b. wt.; Closed circles: 75 mg/kg b. wt.; Open circles: 100 mg/kg b. wt.; Line: 125 mg/kg b. wt.; Open diamonds: 150 mg/kg b. wt.; Closed stars: 175 mg/kg b. wt.; Open stars: 200 mg/kg b. wt.; Closed hexagon: 225 mg/kg b. wt. N=10

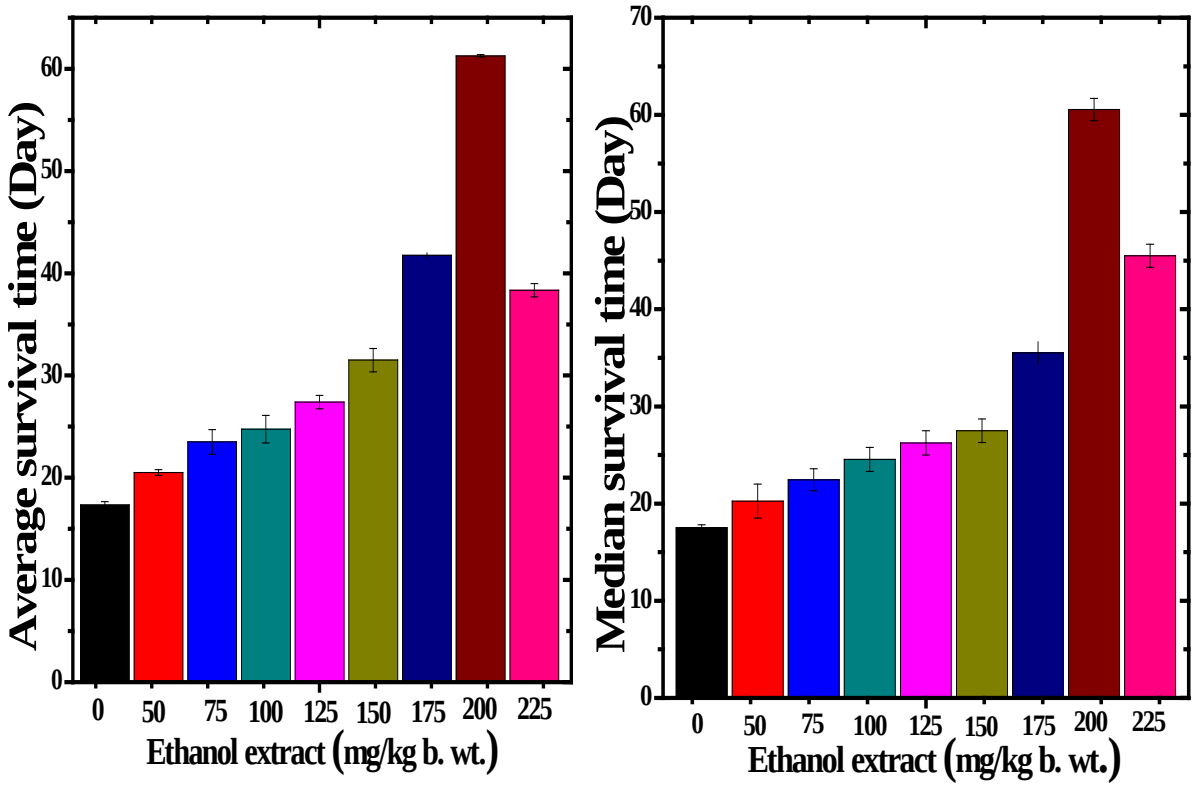

Figure 3: Effect of ethanol extract of *Colocasia gigantea* in Dalton's lymphoma ascites bearing mice on the tumor response assessment based on average survival time (AST) and median survival time (MST). N=10.

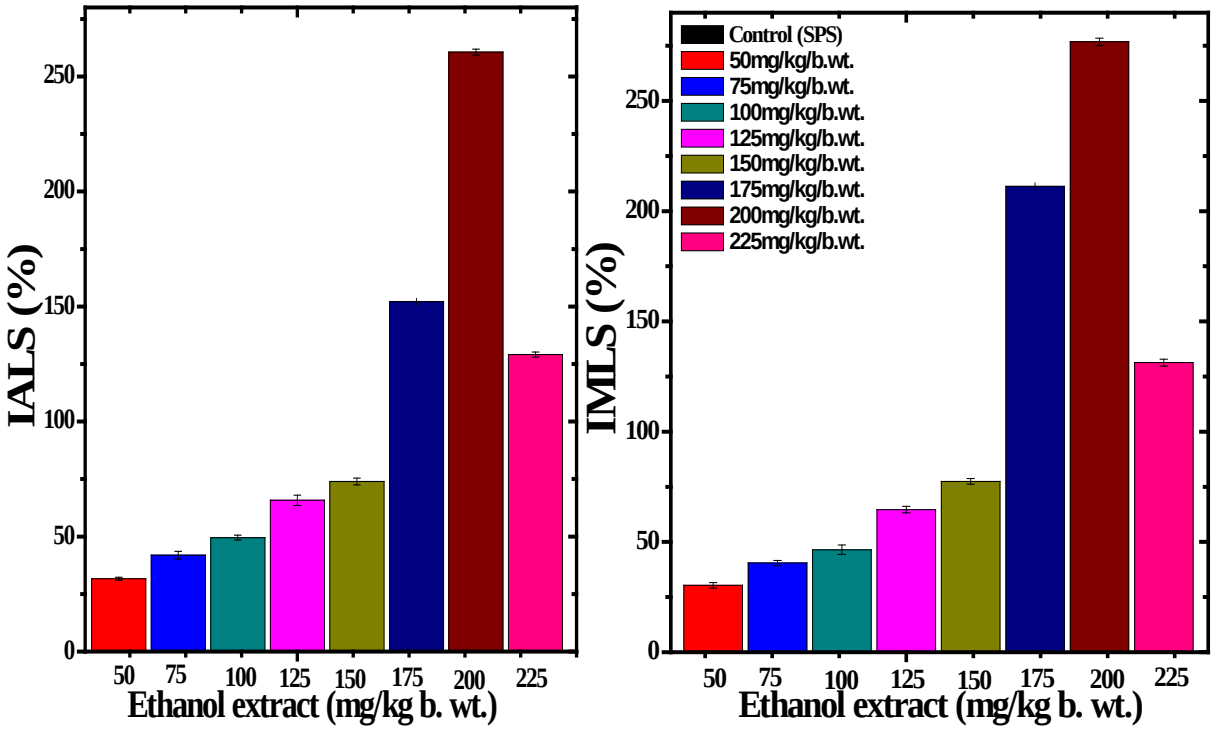

Figure 4: Effect of ethanol extract of *Colocasia gigantea* in Dalton's lymphoma ascites bearing mice on the tumor response assessment based on increase in average life span (% IALS) and increase in mean life span (%IMLS). N=10.

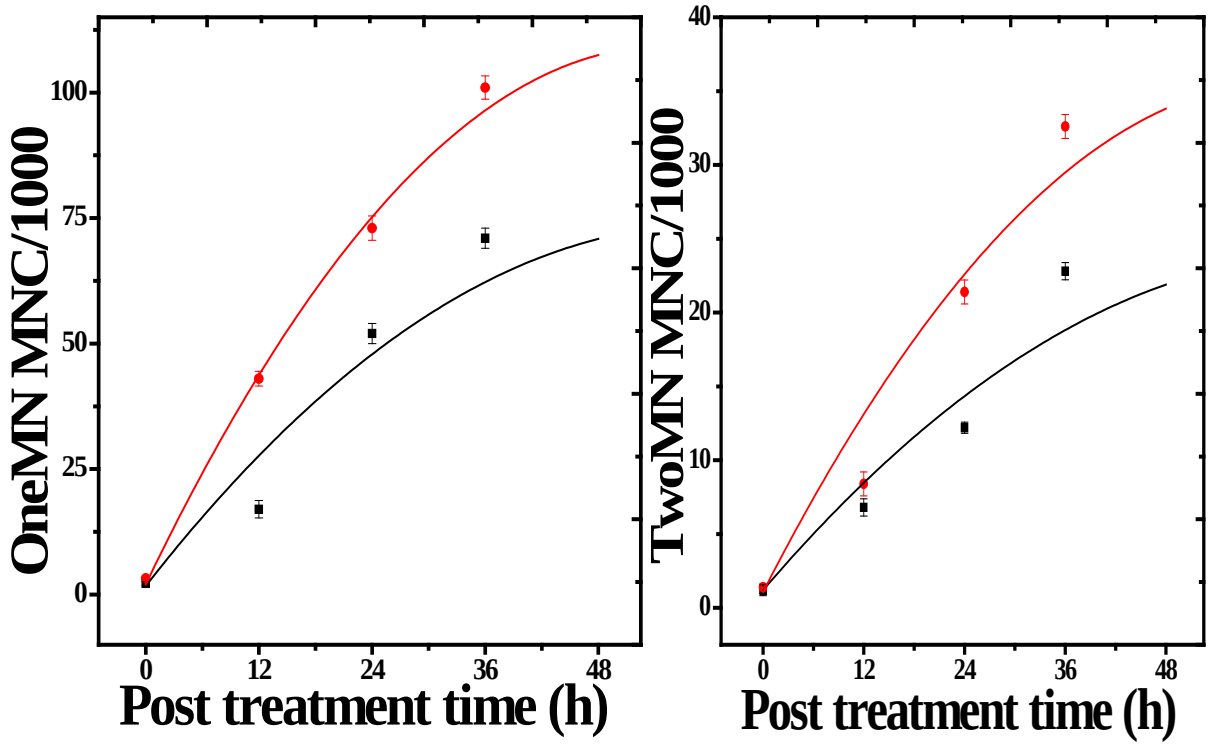

Figure 5(a): Frequency of micronucleated mononucleate cells in Dalton's lymphoma ascites bearing mice treated with 200mg/kg b.wt. ethanol extract of *Colocasia gigantea* (CGE) or 0.5mg/kg b.wt.

doxorubicin (DOX) at different post treatment times. Above left: One micronucleus in mononucleated cells. Above right: Two micronucleus in mononucleated cells. Squares: CGE and Circles: DOX. N=5.

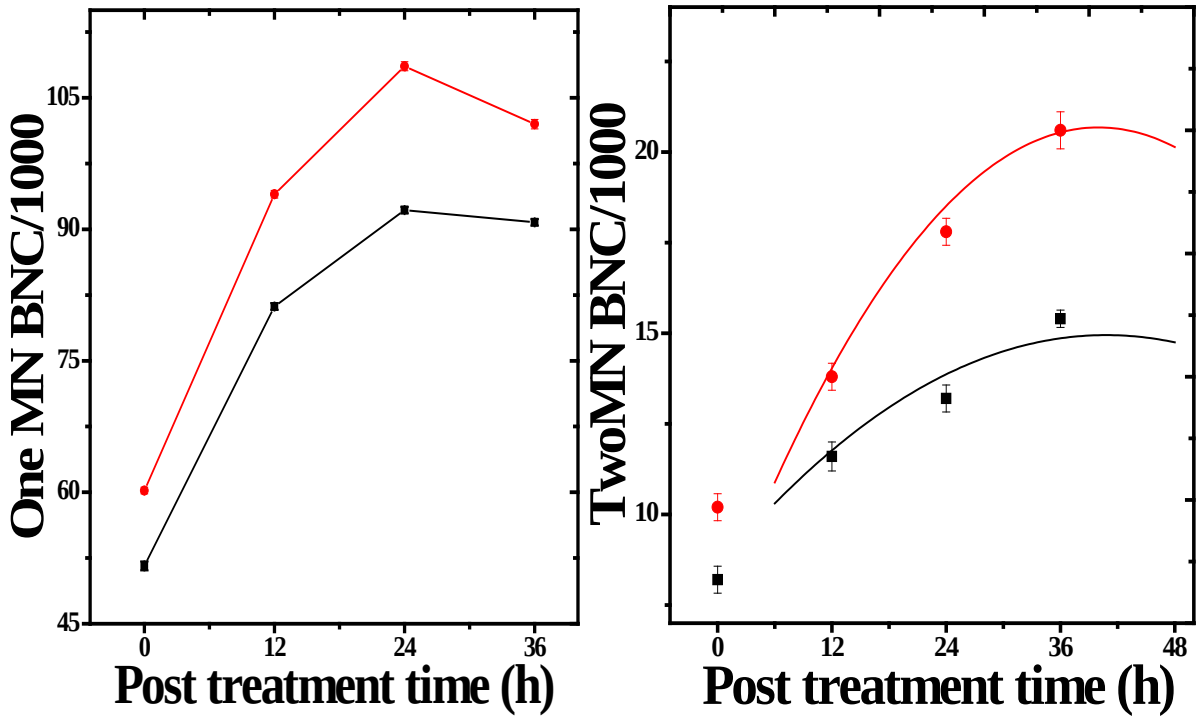

Figure 5(b): Frequency of micronucleated binucleate cells in Dalton's lymphoma ascites bearing mice treated with 200 mg/kg b. wt. ethanol extract of *Colocasia gigantea* (CGE) or 0.5mg/kg b. wt. doxorubicin (DOX) at different post treatment times.Above left: One micronucleus in binucleated cells. Above right: Two micronucleus in binucleated cells. Squares: CGE and Circles: DOX. N=5.

Anticancer activity

Dalton's lymphoma transplanted mice developed tumour rapidly with no signs of regression and all the untreated tumorized mice died within 18-20 days (Table 7). The AST and MST for this group were found to be 17.33 and 17.5 days, respectively (Table 5; Figure 4).

The treatment of tumorized mice with 50, 75, 100, 125, 150, 175, 200 or 225 mg/kg body weight CGE caused a significant ($p<0.001$) rise in the number of survivors when compared to spontaneous control group (Table 7; Figure 2). A 30% rise in survival was observed in animals treated with 50 mg/kg CGE by 20 days. Time of survival increased with increasing dose up to 175 mg/kg where 20% animals survived up to 48 days and all animals succumbed to death after 54 days (Table 7). A further increase in CGE dose elevated the survival of animals up to 50% until 60 days (Table 7). The AST of 44 and MST of 60.5 days were reported for 200 mg/kg CGE leading to an IMLS of 211.27% and an IALS of 152.16%, respectively (Table 8; Figure 3).

Micronucleus Assay

The frequency of micronuclei bearing mononucleate (MNMNC) and binucleate cells (MNBNC) with one and two micronuclei has been represented separately (Table 9, Figure 4(a) & 4 (b)). Treatment of Dalton's lymphoma bearing mice with CGE or DOX showed a time dependent rise in the frequency of micronuclei ($p<0.001$) in a dose dependent manner up to 36 h post-drug treatment in both the mononucleate and binucleate cells (Figure 4). The CGE treatment

not only induced mononucleated and binucleated cells bearing one micronuclei but also the cells bearing two micronuclei (Figure 4).

Table 6: Change in body weight of Dalton's lymphoma bearing Swiss albino mice.

Dose (mg/ kg. b.wt)	Body weight (g)±SEM												
	Post tumour transplanted time (day)												
	0	3	6	9	12	15	18	21	24	28	30	33	36
0	25.97± 0.62	26.34± 0.67	27.4± 0.59	28.55± 0.59	29.36± 0.56	30.39± 0.61	30.26± 0.96						
50	25.97± 0.62	26.34± 0.67	27.4± 0.59	28.55± 0.59	29.36± 0.56	30.39± 0.61	31.06± 0.58	31.42± 0.59	31± .62				
75	29.66± 0.95	30.04± 0.98	31.2± 0.94	32.12± 0.92	33.21± 0.95	33.84± 0.90	34.78± 0.82	35.25± 0.91	34.37± 0.55	34.5± 0.51	29.66± 0.95	30.04± 0.98	31.2±0. 94
100	29.82± 0.67	30.46± 0.65	31.2± 0.65	33.01± 0.64	32.7± 0.74	33.4± 0.73	33.6± 0.71	33.52± 0.64	33.97± 0.64	34.77± 0.70	34.2±0. 51	35.15± 0.31	35.7±0 ±.67
125	29.78± 0.39	30.37± 0.36	31.3± 0.41	32.21± 0.38	33.53± 0.46	34.71± 0.59	35.86± 0.76	37.01± 0.89	36.42± 0.82	37.52± 0.90	37.2±1. 09	29.78± 0.39	30.37± 0.36
150	29.29± 0.31	30.03± 0.30	31.16± 0.32	32.42± 0.45	32.42± 0.48	34.25± 0.48	34.72± 0.39	35.67± 0.40	36.62± 0.39	38.1±0. 47	39.36± 0.51	40.36± 0.61	40.7±0. 51
175	28.85± 0.33	29.11± 0.33	30.11± 0.44	31.3± 0.37	31.94± 0.41	33±0.4 5	33.51± 0.36	34.37± 0.38	34.98± 0.24	35.82± 0.23	36.75± 0.32	37.9±0. 42	38.91± 0.51
200	28.73± 0.26	30± 0.18	31.01± 0.27	32.18± 0.28	33.82± 0.36	35.5± 0.52	37.5± 0.52	39.2±0. 59	41.2±0. 29	42.2±0. 25	44.05± 0.02	45±0.2 6	45.6±0. 18
225	29.29± 0.31	29.58± 0.30	30.67± 0.18	31.87± 0.30	32.87± 0.37	33.63± 0.35	34.5± 0.35	35.48± 0.26	36.66± 0.31	37.22± 0.27	38.14± 0.32	39.02± 0.43	39.67± 0.60

N=10 for each dose

Table 7: Effect of ethanol extract of *Colocasia gigantea* on Survival of Dalton's lymphomas ascites bearing mice treated with various doses administered intraperitoneally.

Post tumor transplant time (day)	SPS (Control)	Survival (%)							
		Ethanol Extract (mg/kg b. wt.)							
		50	75	100	125	150	175	200	225
0	100	100	100	100	100	100	100	100	100
18	30	90	90	60	90	50	80	100	90
20	0	90	90	60	90	50	80	100	90
22	0	70	80	40	80	40	80	90	60
24	0	10	40	40	40	40	70	90	60
26	0	0	40	40	40	40	70	90	60
28	0	0	20	20	40	30	70	80	50
30	0	0	0	20	20	30	70	80	50
32	0	0	0	20	2 0	30	70	80	50
38	0	0	0	0	10	2 0	70	70	40
40	0	0	0	0	0	0	40	70	20
44	0	0	0	0	0	0	40	60	20
46	0	0	0	0	0	0	20	60	10
48	0	0	0	0	0	0	20	60	0
54	0	0	0	0	0	0	0	60	0
60	0	0	0	0	0	0	0	50	0
120	0	0	0	0	0	0	0	50	0

N=10.

Apoptosis Assay

The administration of CGE or DOX induced apoptosis in Dalton's lymphoma cells as early as 12 h post drug treatment in a time dependent manner (Figure 5). The number of apoptotic cells in CGE treated DLA cells significantly ($p<0.001$) increased when compared to concurrent control group at all the post CGE treatment times and maximum number of apoptotic cells were reported at 36 h post-treatment (Table 10). This increase in apoptotic index was 14 folds higher for the all the post CGE treatment times (Table 10).

Biochemical Assays

Glutathione content

The treatment of DLA mice with 200 mg/kg b. wt. CGE led to a significant decrease in the glutathione contents since 2 h post treatment and it continued to decline up to 24 h post treatment, where the reduction in GSH concentrations was highest (Figure 6). The difference in this alleviation in GSH contents between 8, 12 and 24 h was non-significant (Table 11). The CGE treatment reduced the GSH contents comparable to DOX treatment (Table 11).

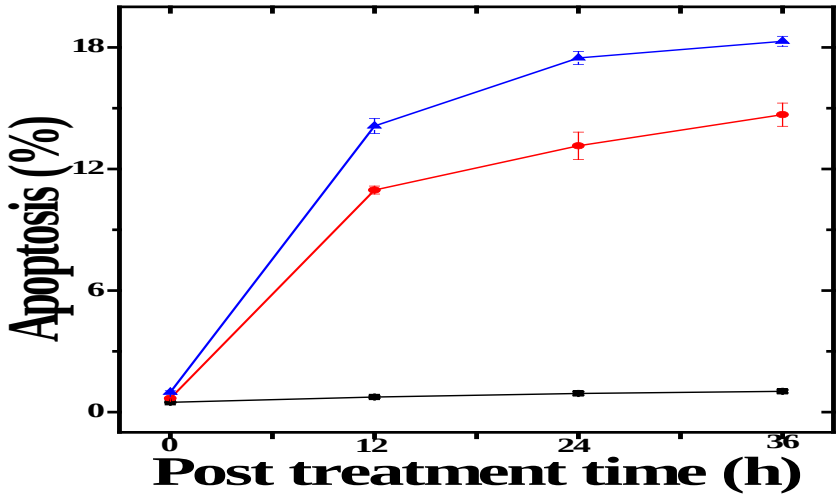

Figure 6: Apoptotic index in Dalton's lymphomas ascites bearing mice treated with 200 mg/kg b. wt. ethanol extract of *Colocasia gigantea* (CGE) or 0.5mg/kg b. wt. doxorubicin (DOX) at different post treatment times. N=5. Squares:SPS; Circle: CGE and Triangles:DOX.

Glutathione - S – Transferase (GST)

The GST activity declined significantly ($p<0.001$) in the DLA mice treated with 200 mg/kg b. wt. CGE (Table 12). The GST activity showed a time dependent decline and the maximum decline was found at 24 h post CGE treatment (Figure 7). The DOX treatment also showed a similar decline in GST activity (Figure 7).

Catalase (CAT) activity

Administration of CGE and DOX led to a gradual and time dependent decline in the catalase activity until 24 h post treatment (Figure 8), where it was 1.4 fold lower than the SPS

treatment (Table 13). The decline in the GST activity was significant ($p<0.001$) when compared to negative SPS treatment (Table 13).

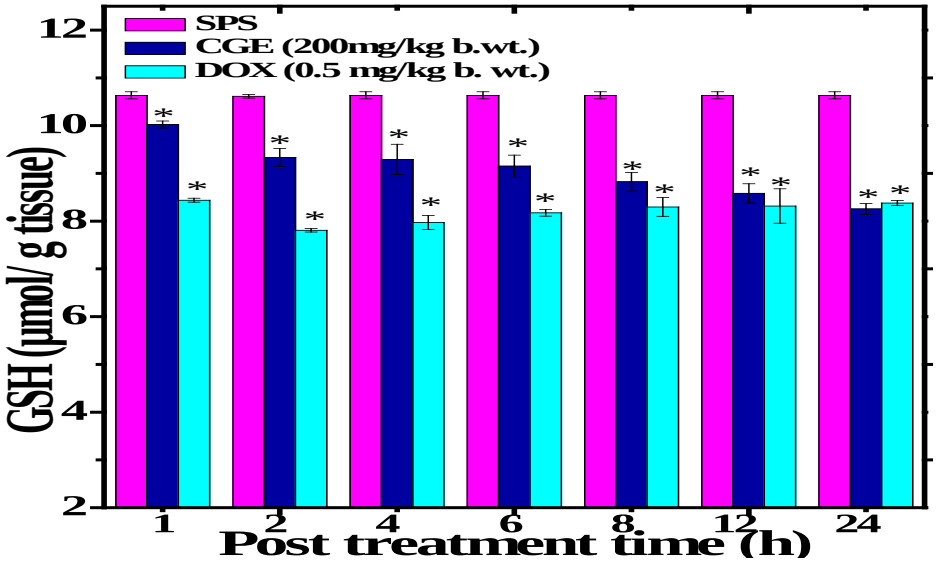

Figure 7: Alteration in the Glutathione contents in mice bearing Dalton's lymphoma ascites treated with 200 mg/kg b. wt. *Colocasia gigantea* extract (CGE) or doxorubicin (DOX). *p<0.001 when treatment are compared with control (SPS) group. No symbol= no significance. N=10.

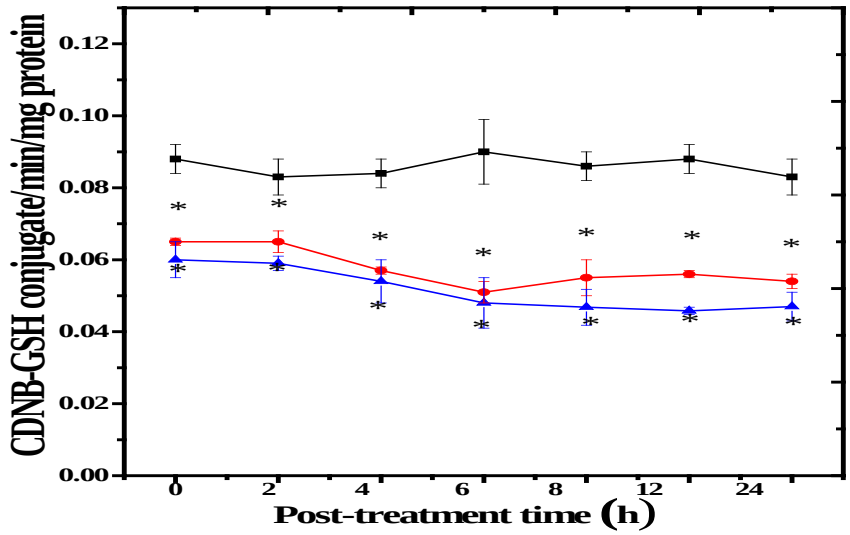

Figure 8: Alterations in the glutathione-s-transferase (GST) activity in Dalton's lymphoma ascites bearing mice treated with 200 mg/ kg b. wt. of *Colocasia gigantea* (CGE) or doxorubicin (DOX). *p<0.001 when treatment are compared with control (SPS) group. No symbol= no significance. Squares:SPS; Circle: CGE and Triangles:DOX. N=10.

Superoxide dismutase (SOD) activity

The SOD activity decreased in a time dependent manner in the DLA mice treated with CGE or DOX treatment until 6 h post treatment where a greatest reduction in SOD activity was observed after CGE administration (Figure 9). This decline was approximately 2.3 and 3 fold for CGE and DOX treatment, respectively when compared to negative SPS control at 6 h post

treatment (Table 14). The SOD activity increased with time after 6 h but did not reach to negative control level even at 24 h post treatment where it was 2 fold lower (Table 14).

Table 8: Effect of ethanol extract of *Colocasia gigantea* on Dalton's lymphoma ascites bearing mice and the tumor response assessment based on median survival time (MST) and average survival time (AST).Increase in mean life span (% IMLS) and increase in average life span (% IALS).

Treatment	Dose (mg/kg.b.wt.)	MST	AST	IMLS (%)	IALS (%)
Control (SPS)	0	17.5±0.33	17.33±0.32	0.05±0.00	0.05±0.00
Ethanol	50	20.25±1.75*	20.5±0.28*	30.3±1.24#	31.63 ±0.65#
	75	22.45±1.13*	23.5±1.21*	40.42±1.18#	41.88±1.68#
	100	24.55±1.24*	24.75±1.35*	46.48±2.11#	49.53±1.055#
	125	26.25±1.25*	27.4±0.65*	64.67 ±1.46#	65.76±2.24#
	150	27.5±1.21*	28.5±1.14*	66.47±1.24#	68.94 ±1.44#
	175	35.5±1.15*	36.75±0.25*	131.33±1.46#	129.12±1.27#
	200	60.55±1.15*	44±0.12*	211.27±1.69#	152.16 ±1.29#
	225	45.5±1.18*	58.22±0.65*	276.79 ±1.58#	260.59 ±1.09#

N=10, **p<0.001, #p<0.0001 when treatment are compared to control group.*

Table 9: Frequency of micronuclei in Dalton's lymphoma ascites bearing mice treated with 200mg/kg b.wt. ethanol extract of *Colocasia gigantea* (CGE) or 0.5 mg/kg b. wt. doxorubicin (DOX) at different post treatment times.

Cell type	Post treatment time (h)	Micronucleated cells/1000±SEM								
		SPS			CGE 200 mg/kg b. wt.			DOX 0.5 mg/kg b. wt.		
		One MN	Two MN	Total	One MN	Two MN	Total	One MN	Two MN	Total
Mononucleated cell	0	1.8±0.37	0.1±0.37	2.6±0.51	2.2±0.37	1.2±0.37	1.7±0.34	3.2±0.20	1.4±0.24	2.3±0.12
	12	4.2±0.37	1.4±0.51	5.6±1.51	17±1.73	6.8±o.58	22.8±0.37	43±1.45	8.4±0.81	45.6±0,75
	24	5.4±0.51	1.6±0.40	7±0.70	52±2.0	12.2±0.37	58.2±1.02	73±2.42	21.4±0.81	88.8±1.24
	36	6.4±0.24	1.8±0.49	8.2±0.37	71±2.0	22.8±0.58	91±0.95	101±2.31	32.6±0.81	119.8±0.91
		One BN	**Two BN**	**Total**	**One BN**	**Two BN**	**Total**	**One BN**	**Two BN**	**Total**
Binucleated cell	0	4.2±0.37	0.8±0.37	5±0.89	51.6±0.51*	8.2±0.37*	59.8±0.86	60.2±0.37*	10.2±0.37*	71.2±0.66
	12	5.2±0.37	1.2±0.58	6.4±0.92	81.2±0.37*	11.6±0.4*	92.8±0.73	94±0.44*	13.8±0.37*	107.8±0.8
	24	5.6±0.51	1.4±0.51	7±1.00	92.2±0.37*	13.2±0.37*	105.4±0.75	108.6±0.51*	17.8±0.37*	126.4±0.87
	36	5.8±0.58	1.8±0.49	7.6±1.08	90.8±0.37*	15.4±0.24*	106.2±0.58	102±0.54*	20.6±0.51*	122.6±1.03

****p<0.001when treatment are compared with concurrent control (SPS) group.***
No symbol= no significance. N=5 for each time

Table 10: Apoptotic index in Dalton's lymphoma ascites bearing mice after treatment with 200 mg/kg b. wt. ethanol extract of *Colocasia gigantea* (CGE) or 0.5 mg/kg b. wt. doxorubicin (DOX) at different post treatment times.

Post treatment time (h)	Apoptosis (% ± SEM)		
	SPS	CGE	DOX
0	0.48±0.02	0.68±0.03*	0.98±0.07#
12	0.74±0.05	10.96±0.19#	14.12±0.36#
24	0.92±0.08	13.14±0.67#	17.48±0.31#
36	1.02±0.07	14.68±0.57#	18.8±0.24#

p<0.05, #p<0.001 when treatment are compared with concurrent control (SPS) group.

No symbol= no significance. N=5 for each time

Table 11: The effect of 200 mg/kg b. wt. ethanol extract of *Colocasia gigantea* (CGE) or Doxorubicin (DOX) on the glutathione contents in mice bearing Dalton's lymphoma ascites at different post treatment times.

Post treatment time (h)	Glutathione (GSH) (µM/mg protein), Mean ± SEM		
	SPS	CGE	DOX
0	10.63±0.07	10.02±0.07*	8.43±0.04*
2	10.61±0.03	9.33±018*	7.81±0.03*
4	10.63±0.07	9.29±0.31*	7.97±0.14*
6	10.63±0.07	9.15±0.23*	8.17±0.07*
8	10.61±0.03	8.82±0.19*	8.30±0.19*
12	10.63±0.07	8.58±0.2*	8.32±0.36*
24	10.63±0.07	8.26±0.11*	8.38±0.05*

p<0.001when treatment are compared with concurrent control (SPS) group.

No symbol= no significance. N=10 for each time

Table 12: The effect of 200 mg/kg b. wt. ethanol extract of *Colocasia gigantea* (CGE) or doxorubicin (DOX) on the glutathione-s-transferase activity in mice bearing Dalton's lymphoma ascites at different post treatment times.

Post treatment time (h)	Glutathione-S-Transferase (U/mg protein), Mean ± SEM		
	SPS	CGE	DOX
0	**0.088±0.004**	**0.065±0.001***	**0.06±0.005***
2	**0.083±0.005**	**0.065±0.003***	**0.059±0.002***
4	**0.084±0.004**	**0.057±0.001***	**0.054±0.006***
6	**0.09±0.009**	**0.051±0.003***	**0.048±0.007***
8	**0.086±0.004**	**0.055±0.005***	**0.0468±0.005***
12	**0.088±0.004**	**0.056±0.001***	**0.0458±0.001***
24	**0.083±0.005**	**0.054±0.002***	**0.047±0.004***

p<0.001 when treatment are compared with concurrent control (SPS) group.

No symbol= no significance. N=10 for each time

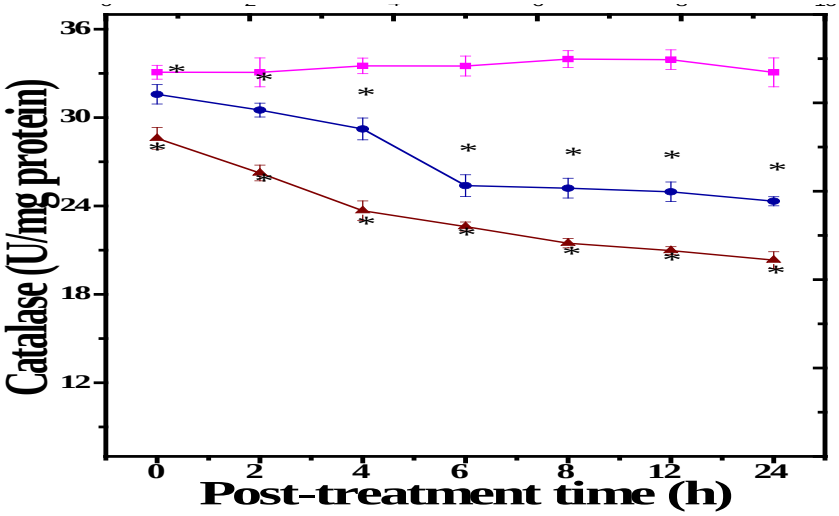

Figure 9: Alterations in the catalase (CAT) activity of Dalton's lymphoma ascites bearing mice treated with 200 mg/ kg b. wt. of *Colocasia gigantea* (CGE) or doxorubicin (DOX). *p<0.001 when treatment are compared with control (SPS) group. No symbol= no significant. Squares:SPS; Circle: CGE and Triangles:DOX. N=10.

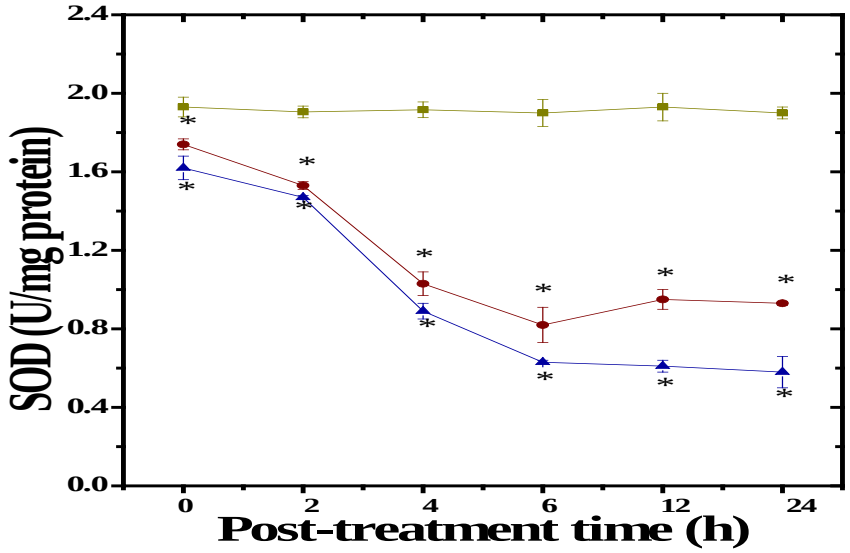

Figure 10: Alterations in the Superoxide dismutase (SOD) activity of Dalton's lymphoma ascites bearing mice treated with 200 mg/ kg body weight of *Colocasia gigantea* (CGE) or doxorubicin (DOX). *p<0.001 when treatment are compared with control (SPS) group. No symbol= no significant. Squares:SPS; Circle: CGE and Triangles:DOX. N=10

Lipid peroxidation (LOO)

Treatment of DLA mice with 200 mg/kg b. wt. CGE led to 3 fold elevation in the lipid peroxidation as early as 1 h post –treatment (Table 15) when compared to SPS group. Increase in assay time resulted in a further rise in LOO and 3.6 fold elevation was recorded at 6 h post treatment in the CGE group (Table 15). The LOO increased in a time dependent manner up to 6 h post treatment in both CGE and DOX treated group and started to increase gradually until 24 h post treatment (Figure 10) where LOO was still higher than the SPS treatment (Table 15).

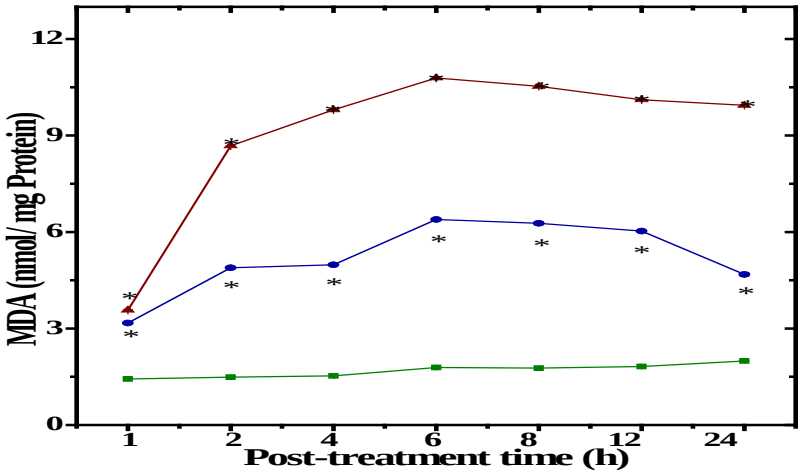

Figure 11: Alterations in the lipid peroxidation (LOO) in the Dalton's lymphoma ascites bearing mice treated with 200 mg/ kg b. wt. of *Colocasia gigantea* (CGE) or doxorubicin (DOX). *p<0.001 when treatment are compared with control (SPS) group. No symbol= no significant. Squares:SPS; Circle: CGE and Triangles:DOX. N=10

Table 13: The effect of 200 mg/kg b. wt. ethanol extract of *Colocasia gigantea* (CGE) or doxorubicin (DOX) on the catalase activity in mice bearing Dalton's lymphoma ascites at different post treatment times.

Post treatment (h)	Catalase (CAT) (U/mg protein), Mean ± SEM		
	SPS	CGE	DOX
0	33.08 ± 0.47	31.58 ± 0.66	28.59 ± 0.74*
2	33.07 ± 0.98	30.51 ± 0.47	26.24 ± 0.53*
4	33.51 ± 0.53	29.23 ± 0.74*	23.68 ± 0.66*
6	33.50 ± 0.68	25.38 ± 0.74*	22.60 ± 0.31*
8	33.93 ± 0.57	25.21± 0.67*	21.47 ± 0.31*
12	33.93 ± 0.67	24.96 ± 0.67*	20.97± 0.27*
24	33.07 ± 0.98	24.32 ± 0.31*	20.32± 0.57*

p<0.001 when treatment are compared with concurrent control (SPS) group.
No symbol= no significance. N=10 for each time

Table 14: The effect of 200 mg/kg b. wt. ethanol extract of *Colocasia gigantea* (CGE) or doxorubicin (DOX) on the superoxide dismutase activity in mice bearing Dalton's lymphoma ascites at different post treatment times.

Post treatment time (h)	Superoxide dismutase (SOD) (U/mg protein), Mean ± SEM		
	SPS	CGE	DOX
0	1.93 ± 0.05	1.74 ± 0.028 *	1.62 ± 0.06*
2	1.905 ± 0.03	1.53 ± 0.02*	1.47 ± 0.01*
4	1.916 ± 0.04	1.03 ± 0.06*	0.89 ± 0.04*
6	1.90 ± 0.069	0.82 ± 0.09*	0.63 ± 0.01*
12	1.93 ± 0.07	0.95 ± 0.05*	0.61 ± 0.03*
24	1.90 ± 0.03	0.93 ± 0.01*	0.58 ± 0.02*

p<0.001when treatment are compared with concurrent control (SPS) group.

No symbol= no significance. N=10 for each time

Table 15: Alterations in the Lipid peroxidation in mice bearing Dalton's lymphoma treated with *Colocasia gigantea* extract (CGE) and Doxorubicin (DOX).

Post treatment time (h)	Lipid Peroxidation (nmol/mg Protein), Mean±SEM		
	SPS	CGE	DOX
0	1.43 ± 0.012*	3.17 ± 0.005*	3.57 ± 0.017*
2	1.49 ± 0.01*	4.89 ± 0.02*	8.68 ± 0.015*
4	1.53 ± 0.03*	4.98 ± 0.01*	9.80 ± 0.028*
6	1.79 ± 0.02*	6.39 ± 0.02*	10.79 ± 0.03*
8	1.77±0.03*	6.27±0.05*	10.53±0.03*
12	1.82 ± 0.01*	6.03 ± 0.01*	10.11 ± 0.02*
24	1.99 ± 0.02*	4.68 ± 0.05*	09.94 ± 0.02*

p<0.0001when treatment are compared with concurrent control (SPS) group.

No symbol= no significance.

N=10 for each time

Liver and Kidney function tests

The intraperitoneal administration of CGE (200 mg/kg b. wt.) for consecutive 9 days did not significantly alter aspartate aminotransferase (AST), and alanine aminotransferase (ALT) in the liver and creatinine and uric acid in the kidney of mice (Table 16 and Figure 11). Therefore, the treatment of 200 mg/kg body weight of CGE did not cause undesirable effect on the liver.

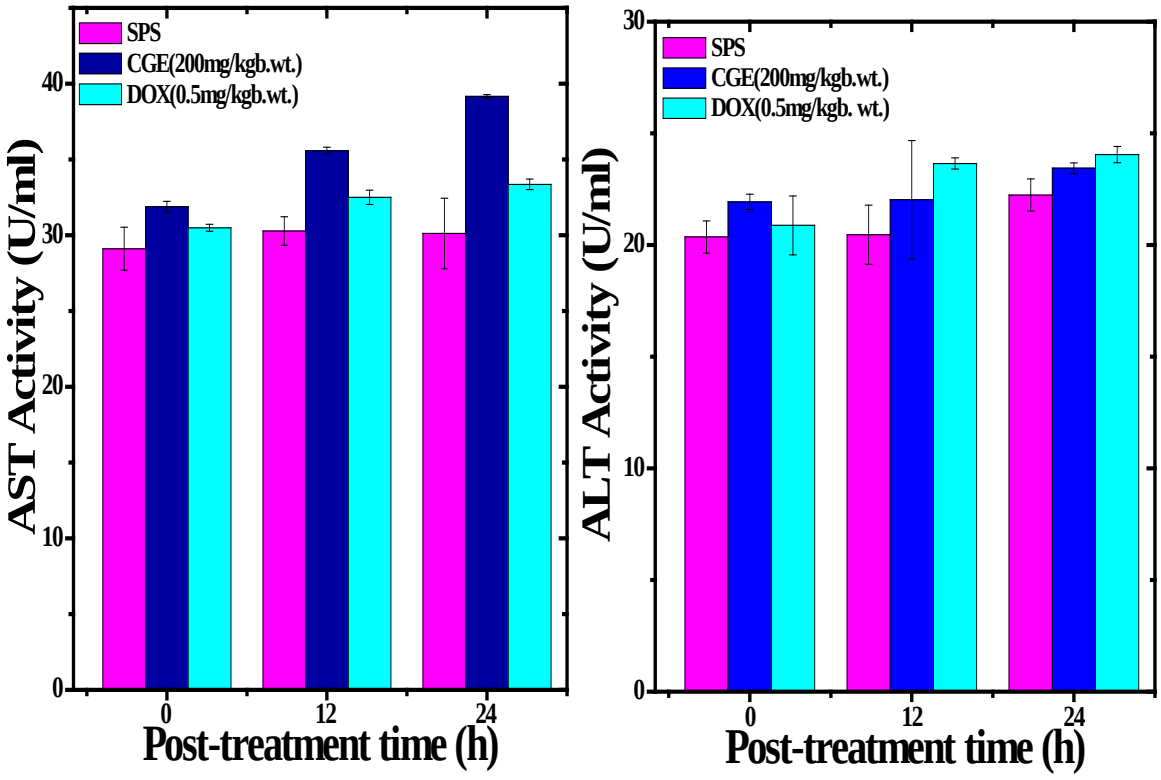

Figure 12(a): The alteration in the liver function by 200 mg/kg b. wt. *Colocasia gigantea* (CGE) or 0.5 mg/kg b. wt. doxorubicin DOX in Dalton's lymphoma ascites bearing mice. N=10

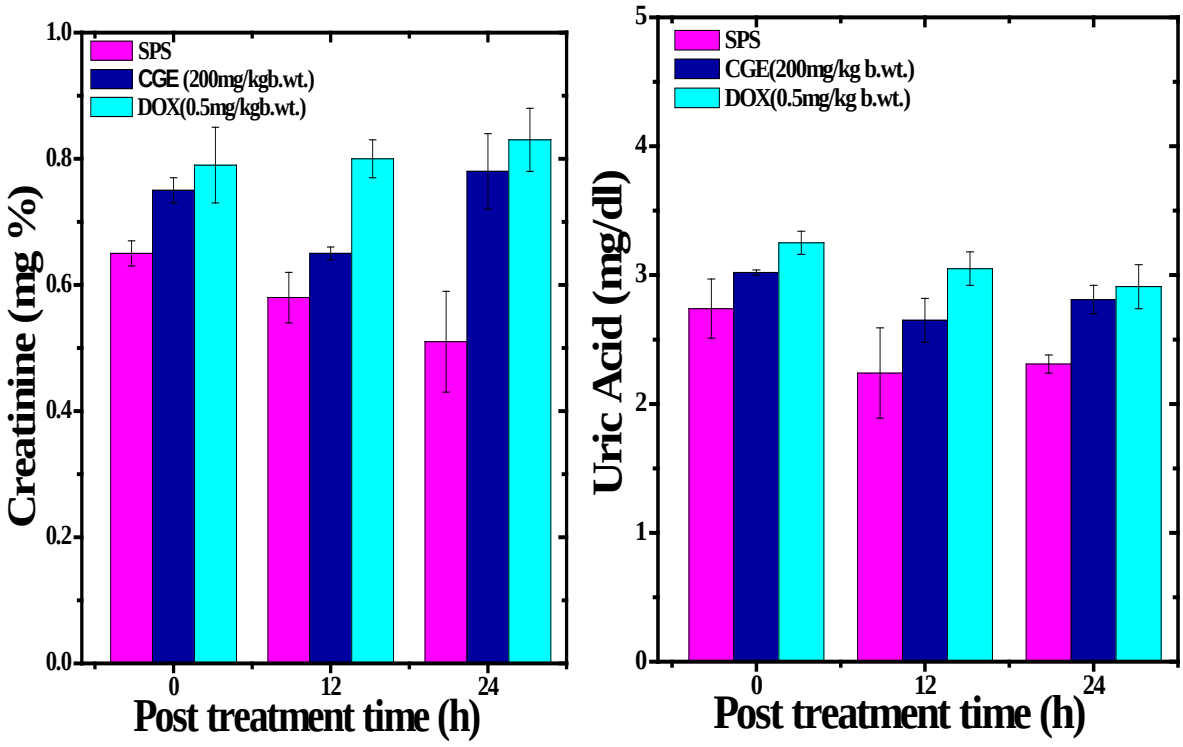

Figure 12(b): The alteration in the kidney function by 200 mg/kg b.wt. *Colocasia gigantea* (CGE) or 0.5 mg/kg b. wt. doxorubicin DOX in Dalton's lymphoma ascites bearing mice. N=10.

Table 16: Effect of *Colocasia gigantea* extract (CGE) and Doxorubicin (DOX) on the liver and kidney function of Dalton's lymphoma bearing mice.

Treatment	Dose (mg/kg.b.wt.)	Post treatment time (h)	Liver function test		Kidney function test	
			AST (U/ml)	ALT (U/ml)	Creatinine (mg%)	Uric acid (mg/dl)
Control (SPS)	0	0	29.11±1.42	18.36±0.72	0.75±0.02	2.74±0.23
		12	29.28±0.94	19.46±1.32	0.58±0.04	2.24±0.35
		24	29.12±2.32	22.24±0.72	0.51±0.08	2.31±0.07
CGE	200	0	33.88±0.35	23.93±0.34	0.75±0.02	3.02±0.02
		12	37.58±0.23	21.03±2.65	0.60±0.01	2.65±0.17
		24	38.16±0.12*	23.44±0.24	0.85±0.06	2.81±0.11
DOX	0.5	0	31.49±0.23	21.88±1.32	0.79±0.06	3.65±0.09
		12	30.50±0.47	24.65±0.25	0.86±0.03*	3.76±0.13*
		24	35.36±0.35*	23.81±0.36	0.87±0.05*	3.81±0.17*

p<0.05 when treatment group are compared with control group.
No symbol= no significance. The results are the Mean ± SEM. N=10 for each time

DISCUSSION

Chemotherapy is one of the most preferred modality to treat cancer, especially when patients have metastasis. Despite increased survival and cure rates, chemotherapy increases toxicity in the normal tissues and also rapidly dividing tissues leading to morbidity and mortality [25,26]. The definite therapy to reduce the toxic effects of chemotherapy is not yet available and efforts to reduce adverse toxic side effects without compromising their efficacy to cure

tumors shall continue. Herbal medicines have been practiced in the world since the advent of human history and their scientific evaluation may help to develop new pharmacophores that can be used as modern therapeutic agents to cure cancer. The use of herbal medicines as adjuvant may be reduce the toxic side effects of chemotherapy and increase its efficacy on neoplastic cells at the same time protecting the normal tissue from chemotherapy-induced toxic side effects. The herbal drug may enhance the immune surveillance of normal tissues, which are affected adversely during neoplastic transformation. The inclusion of herbal medicine in chemotherapy may improve the therapeutic index by killing neoplastic cells and reducing the toxicity to normal tissues [27]. The natural products may play an important role by killing neoplastic cells and not allowing the normal cells to transform into the malignant phenotype. The advantage of natural products is that they are natural in origin and hence most biocompatible with minimum side effects in comparison to chemical synthetic products [28]. Therefore, the present study was undertaken to evaluate the ability of *Colocasia gigantea* to kill the Dalton's lymphoma cells transplanted in mice.

Different extracts of *Colocasia gigantea* administered with a single oral dose showed no signs of any toxicity up to 2 g/kg b. wt. in Swiss albino mice hence it can be considered safe orally. However, the intraperitoneal mode of administration revealed significant toxicity, where the chloroform, ethanol and aqueous extracts showed LD_{50} of 625 mg/kg b. wt., 823 mg/kg b. wt. and 710 mg/kg b. wt., respectively. In an earlier study the LD_{50} for interaperitoneal administration was found to be lower than oral admiration for leaf extract of *Blighia unijugata* [29].

Assessment of antitumour activity on Dalton's lymphoma transplanted intraperitoneally nto mice indicated that DLA cells grew rapidly and all the tumorized mice died within 18-20 days with an average survival time (AST) and median survival time (MST) of 17.33 and 17.5 days respectively. Treatment of DLA mice with different doses of CGE led to a rise in the survival of mice in a dose dependent manner and a maximum number of survivors were observed at a dose of 200 mg/kg b. wt. with a 50% tumor free survivors beyond 120 days. The increase in tumor free survivors have been reported for the stem bark extract of *Alstonia scholaris, Aphnamixis polystachya, Ervatamia heyncana, Hygrophila spinosa, Podyphyllum hexandrum, Rubia cordifolia, Tinospora cordifolia* and *Tylophora indica* earlier [30-36].

The infliction of DNA damage is one of the important events to kill tumor cells and many chemotherapeutic agent induce DNA damage to kill neoplastic cells [37]. The ability of ethanol extract of *Colocasia gigantea* to trigger the DNA damage was tested in the tumorized mice and it was found that CGE induced DNA damage as evidenced by the increase in the formation of micronuclei in mononucleated as well as binucleated cells effectively. Treatment of Dalton's lymphoma bearing mice with CGE showed a time dependent elevation in the frequency of micronuclei up to 36 h post treatment. A similar effect has been observed earlier [38-40]. The CGE induced not only one micronuclei but also cells with two micronuclei indicating that it induced complex DNA damage in the form of multiply damaged sites that

would have repressed the DNA damage repair leading to higher cell death. A number of studies have indicated that the cells expressing micronuclei are dying cells and correlation between cell killing and micronuclei has been reported [38-41]. The micronuclei assay provides an indirect measure of DNA damage since the micronuclei arise due to defective cell division, mis-segregation of chromosomes, DNA exchanges and faulty or suppressed DNA repair leading to cell death [39-45]. The formation of DNA DSBs and micronuclei is often the consequence of simultaneous excision repair of damages, wrong base incorporation and failure of the appropriate gap-filling event [46]. This may happen only if the level of DSBs exceeds the repair capacity of dividing cells, which is mainly due to either the misrepair of DSBs by the dysfunctional homologous recombination [47].

The apoptosis induction is a silent form of cell death and many chemotherapeutic agents induce apoptosis to shrink the tumor [48,49]. One of the important cause of cell death by CGE seems to be induction of apoptosis. Treatment of DLA mice with CGE triggered apoptosis in a time dependent manner leading to increased tumor free survivors in the present study. The infliction of DNA damage in the cells by CGE may have triggered a cascade of biochemical and molecular events inducing apoptosis, which was characterized by chromosome condensation, DNA fragmentation, membrane blebbing and formation of apoptotic bodies and cell death [50,51].

The cancer cells are always at higher oxidative stress and a further increase in oxidative stress will stimulate cells to undergo DNA damage and thus it is able to kill tumor cells effectively. The chemotherapeutic agents are known to kill tumor cells by modulating oxidative stress [52,53]. The treatment of tumor bearing mice has reduced the glutathione concentration as increased GSH is involved in resistance to apoptosis and also to chemotherapy, whereas reduced glutathione sensitizes cancer cells to death [54-58]. Glutathione is an important biomolecules synthesized by cells and it plays crucial role in the detoxification, cell differentiation, proliferation and apoptosis however, reduced GSH levels cause oxidative stress and aids in cell death [56,59-61. The cell killing effect of CGE may be due to its ability to reduce glutathione concentration in the tumor cells. Similarly, treatment of Dalton's lymphoma with CGE had a negative effect on the activities of GST, catalase and SOD, which are also involved in resistance to chemotherapy [62-64]. This depletion in their activities may have made tumors cells susceptible to the cytotoxic effect of CGE causing increased tumor free survival in the present study.

The lipid peroxidation is involved in increased oxidative stress and cell death when chemotherapeutic agents come into the contact of cancer cells. Since lipid are integral part of cell membrane and their peroxidation damages the cell membrane thus killing the cells effectively [65,66]. The increase of lipid peroxidation in DLA cells by CGE may have killed the tumor cells by damaging their membrane and inducing damage of proteins and nucleic acids [66].

The mechanism of tumor cell kill by CGE is not well understood. However, employment of multiple putative pathways to kill tumor cells seems to be operational in the present study. The increase in lipid peroxidation may have changed cell membranes and also the important macromolecules like DNA and proteins that in turn may have killed the tumor cells and increased the tumor free survivors in the present study. The CGE has actually increased the DNA damage in both mono and binucleated tumor cells and also induced apoptosis which supports the above contention. The reduction in GSH, GST, catalase and SOD by CGE would have increased the oxidative stress in the DLA cells bringing effective cell killing in the present study. At molecular levels negative modulation of Nrf2, COX-II and NF-κB by CGE may have favored the tumor cell killing as the activation of these genes is involved in failure of tumor therapy [67-69]. Since CGE induced apoptosis it may have stimulated apoptotic cascade by upregulating p53, caspases, Bax and other proteins in the present study. Some other unknown mechanisms may also have contributed to the cytotoxic effect of CGE.

CONCLUSIONS

The CGE killed tumor cells and increased the tumor free survival, which may be due to its ability to induce DNA damage and it increased micronuclei and apoptosis. The apoptosis may have been triggered by the activation of p53, Bax and p21 and caspases. It may have also suppressed the transcriptional activation of NF-κB, COX-II and Nrf2. The elevation in lipid peroxidation and depletion in GSH, GST, catalase and SOD may have played a major role in inducing DNA damage and stimulating apoptotic and non-apoptotic pathways that finally killed the DLA cells and increased the tumor free survivors in CGE treated mice beyond 120 days.

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