"Pharmacological Evaluation of Cocoa on Efficacy and Toxicity of Doxorubicin in Murine Ascites and Solid Tumor"

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For the award of the degree of

Doctor of Philosophy
In the Faculty of Pharmacy

By

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Mrs. Priyanka. Patil

ABSTRACT

Background: Doxorubicin (DOX) is an extensively utilised chemotherapy drug, oxidative stress caused by it can damage the heart, liver, and kidneys. *Theobroma cacao* L. (cocoa) is reported to possess protective efficacy against several chemical-induced organ damages and also anticancer activity. The present study aimed to evaluate the effect of cocoa extract (COE) on doxorubicin-induced organ damage in mice with Ehrlich ascites carcinoma (EAC) by employing *in silico*, *in vitro*, and *in vivo* studies.

Objective:

1. To assess protective activity of cocoa against Doxorubicin induced organ toxicities (Heart, Liver and Kidney) in a mouse bearing ascites & solid tumor

2. To assess Cytotoxic activity of Doxorubicin in combination with cocoa in a mouse bearing ascites & solid tumor

Methodology: To begin, multiple *in silico* methods (from compound target identification to compound-binding-to-target validation) were used with stringent checkpoints to investigate the likely mechanism of action of cocoa for its antioxidant and anticancer activity. The LC-MS technique was used to infer the crude extract metabolite profile. Cytotoxicity of COE in CHO (normal) and EAC (cancer) and A549 (cancer) cell lines was performed. After a mouse survival analysis, the organ-protective effect of COE on DOX-treated animals with EAC-induced carcinoma was investigated.

Results: Cocoa bioactives were found to target 21 molecular pathways *via* targeting 50 protein molecules, in which these compounds highly targeted "EGFR". Hirsutrin exhibited stable contacts with EGFR after a 100 ns MD run. In vitro studies revealed the potent cytotoxicity of COE on cancer cells compared to normal. Interestingly, COE enhanced DOX potency when used in combination. The *in vivo* results revealed reductions in EAC and DOX-induced alterations in mice treated with COE. COE improved the mouse survival time, percentage of life span, antioxidant defence system, improved renal, hepatic, and cardiac function biomarkers and also oxidative stress markers. COE reduced DOX-induced histopathological alterations.

Conclusion: The anti-cancer and antioxidant effects of COE may reduce DOX-induced organ damage without affecting the anticancer effects of DOX. Therefore, COE may be useful as an adjuvant nutritional supplement in DOX cancer therapy.

Keywords: Anticancer; Cardiotoxicity; Cytotoxicity; Cocoa; Doxorubicin; Ehrlich ascites carcinoma; Hepatotoxicity; Molecular docking; Molecular dynamics; Network Pharmacology; Nephrotoxicity; organ toxicity; *Theobroma cacao* Linn;

LISTOFABBREVIATIONS

ALP: Alkaline phosphatase; **ALT**: Alanine transaminase; **ANOVA**: Analysis of variance; **AST**: aspartate aminotransferase; **BE**: Binding energy; **BUN**: Blood urea nitrogen; **CAT**: Catalase; **CHO**: Chinese hamster ovary; **CID**: Compound identification number; **COE**: hydroalcoholic extract of cocoa bean; **CPK-MB**: Creatine phosphokinase-MB; **CYP**: Cytochromes P450; **DLS**: Drug-likeness score; **DOX**: Doxorubicin-treated animal group; **DOPE**: Discrete optimized protein energy; **DPPH**: 2,2-diphenyl-1-picrylhydrazyl; **EAC**: Ehrlich ascites carcinoma; **ECG**: Electrocardiogram; **EDTA**: Ethylenediaminetetraacetic acid; **EGFR**: Epidermal growth factor receptor; **EMT**: Epithelial mesenchymal transition; **FDR**: False discovery rate; **GSH**: Glutathione; **Hb**: Haemoglobin; **HDL**: High-density lipoprotein; **IC_{50}**: Inhibitory concentration; **ICMR-NITM**: Indian Council of Medical Research-National Institute of Traditional Medicine; **ILS**: Increased life span; **KEGG**: Kyoto encyclopedia of genes and genomes; **LDL**: Low-density lipoproteins; **LDH**: Lactate dehydrogenase; **LPO**: Lipid peroxidation; **MCH**: Mean corpuscular haemoglobin; **MCHC**: Mean corpuscular haemoglobin concentration; **MF**: Molecular formula; **mg**: Milligram; **mL**: Milliliter; **MST**: Mean survival time; **NO**: Nitric oxide; **PCV**: Packed Cell Volume; **PDB**: Protein data bank; **pH**: Potential of hydrogen; **RBC**: Red blood cells; **Pt**: Pretretment; **RMSD**: Root mean square deviation; **RMSF**: Root-mean-square fluctuation; **ROS**: Reactive oxygen species; **SD**: Standard deviation; **SEM**: Standard error of the mean; **SMILES**: Simplified molecular-input line-entry system; **SNP**: Sodium nitroprusside; **SOD**: Superoxide dismutase; **STRING**: Search tool for the retrieval of interacting genes/proteins; **TG**: Triglycerides, **WBC**: White blood cells.

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1. INTRODUCTION

1.1. Background

DOXORUBICIN (DOX) was first used to treat cancer way back from 1969 and has demonstrated strong antitumor activity. DOX is proven to have cytotoxic effects on cancer cells and also induces toxic effects on various organs due to DNA intercalation and lipid-binding activity on cell membranes. Also, it has been proposed that dox-induced apoptosis is also responsible for its therapeutic effect, toxicity, or both.[1] Despite its high antitumor efficacy, the usage of dox in cancer chemotherapy has been restricted due to its ADR (adverse effects) (heart, liver, kidney, lungs, testis, spleen, and blood cells).[2,3] Dox chemotherapy employs chemicals to halt the growth and destruction of malignant cells, even in locations far from the tumor's origin. However, it does not distinguish between cancerous and non-cancerous cells, and therefore it acts on all the fast-multiplying cells in the body.[4] The severity of toxicity is directly proportional to the cumulative doses of dox, and it ranges from subclinical myopathy to cardiac infarction, and it may cause the death of the patients in the most severe reports.[5] Dox doses up to 400 mg/m^2 may raise the risk of cardiotoxicity by 5%; 550 mg/m^2, however, which is recommended as the upper limit for treatment, may increase the risk by 26%. It has been reported that, after several weeks of dox treatment, life-threatening chronic ill effects are frequently observed with elevated levels of serum enzymes such as cardiac-CPK-MB, LDH, hepatic-ALT, AST, ALP, renal-creatinine, and BUN.[2,6,7] Although the mechanisms underlying severe dox cytotoxicity are not completely understood, reactive oxygen species (ROS) are thought to be important factors responsible for dox toxicity, and the events that control this oxidative damage are highly valued. NADPH-cytochrome P450 converts dox to semiquinone free radicals, which in turn leads to the production of

ROS(superoxide anions, hydrogen peroxide, and hydroxyl free radicals), resulting in membrane lipid peroxidation (LPO).[8] Because of the increasing health burden and prevalence of doxorubicin-induced cardiotoxicity around the world, it is essential to find a therapeutic agent that protects against these serious side effects to increase safety without compromising the efficacy of dox chemotherapy.[9,10] Previous reports have proven that combining chemotherapy with antioxidant agents can improve the efficacy of cancer chemotherapeutic agents.[11]

Indian traditional medicines have a long history and a large collection of medicinal herbs, which are being used to treat many ailments like communicable and non-communicable diseases. Herbal medicines are being used extensively and play an important role in cancer chemotherapy, and approximately 60 percent of the cancer chemotherapy agents that are currently available are derived from herbal origins.[12] The major classes of herbal anticancer drugs currently used in the market, such as alkaloids like vinblastine, vincristine, and vinorelbine, are derived from *Catharanthus, epipodophyllotoxins* like etoposide, etoposide phosphate, from *Podophyllum,* and taxanes like paclitaxel, docetaxel, are derived from Taxus and camptothecin derivatives are derived from *Camptotheca acuminate.* Much of the recent research is focused on cancer therapeutics to identify new therapies or additional alternatives to reduce the toxic effects of conventional chemotherapies with the same or better therapeutic effect.[13,14] In this regard, one can consider conceptualising the broad pharmacological spectral activity in cancer management by "neutralizing the ROS system and increasing the pharmacological threshold of established chemotherapeutic agents," which can be accomplished by utilising traditional medicines, which are rich in bioactive principles for multiple pharmacological activities with broad biological processes.[15]

In this context, the herb *Theobroma cacao* L., commonly referred to as cocoa, from the family Sterculiaceae, has its origin in Central America. Its seeds have been documented to act across a wide range of biological spectra. The primary phytoconstituents are flavonoids, polyphenols, and alkaloids.[16]Additionally, cocoa has been reported for free radical scavenging,[17]anti-inflammatories,[18] anti-cancer,[12,19] hepatoprotective activities,[20] and nephroprotective activity.[21] Cocoa leaf and bark extract proved to have a protective effect against anthracycline dox-induced oxidative stress and organ failure. (hepatotoxicity, nephrotoxicity, and splenotoxicity).[22,23] It has also been shown to be cytotoxic against cancer cell lines.[19,24]

Although cocoa has been reported to reduce organ toxicities and have anti-cancer activities, its effect along with conventional chemotherapeutics in cancer models has yet to be reported. As a result, the current investigation was carried out to examine the impact of COE on the multi-organ toxicity of doxorubicin in the EAC mouse model as well as to determine whether it has any anti-cancer effects.

Research gap

Theobroma cacao extract exhibits anti-cancer, anti-oxidant as well organ protective activities; this activity in a setting with conventional cancer therapy is not established.

1.2. Justification

The anthracycline derivative doxorubicin is a clinically proven anticancer drug that is widely used to treat various types of cancer. The usage of this drug is limited because its associated organ toxicities especially cardiomyopathies are frequently encountered. Doxorubicin and its metabolites induced oxidative stress due to the generation of free radicals which in turn leads to membrane lipid peroxidation, mitochondrial damage and iron dependence on macromolecular oxidative damages.

While extensive research is underway to find new anti-cancer drugs with more efficacy, there is also an urgent need to address issues compared to severe adverse side effects associated with currently using anticancer drugs to increase the quality of life of patients.

Theobroma cacao extract is reported to have anti-cancer, anti-oxidant as well organ protective activities; this activity in a setting with conventional cancer therapy is not established.

1.3. Hypothesis

Administration of cocoa extract with doxorubicin to mice bearing ascites or solid tumor could ameliorate the organ toxicities and enhance efficacy of doxorubicin.

2. OBJECTIVES OF THE PROPOSED STUDY

- To assess protective activity of cocoa against Doxorubicin-induced organ toxicities (Heart, Liver and Kidney) in a mouse bearing ascites & solid tumor
- To assess Cytotoxic activity of Doxorubicin in combination with cocoa in a mouse bearing ascites & solid tumor

3. REVIEW OF LITERATURE

3.1. Background

Cancer is a disease that occurs when some cells of the body divide uncontrollably and spread to other body parts, thus hampering the function of healthy cells and altering homeostasis. The balance or imbalance of oncogenes, proto-oncogenes, and tumor suppressor gene expression regulates the proliferation of normal cells and cancer cells genetically. During the events of neoplastic transformation, tumor cells can spread, invade normal tissues, kill healthy cells, and disrupt organ function. The chemotactic factors released by the cancerous cells and the surrounding tissues encourage the development of new blood vessels to carry nutrients and oxygen to these cells through the processes of neovascularization and angiogenesis.[25] In later stages, tumor cells, detached from the central mass and disseminated to the distant regions of the body through lymphatic and blood vessels, leading to metastasis.

3.2. Epidemiology of Cancer

Cancer is a significant public health burden and is responsible for the second leading cause of global mortality. Approximately two crore new cases of different types of cancer and one crore cancer deaths were reported globally in the year 2020. The most frequent type of cancer is breast cancer (11.7%), followed by lung carcinoma (11.4%), bowel cancer (10.0%), prostate cancer (7.3%), and stomach carcinoma (5.6%). Colon cancer (9.4%), stomach cancer (7.7%), breast cancer (6.9%), and liver cancer (8.3%).[26] Lung cancer remains the leading cause of cancer death, accounting for roughly 18% of the total.

3.3. Cancer causes

About 90–95 % of cancer cases are caused by environmental causes (tobacco, infection, radiation, alcoholism, lifestyle, and environmental pollution), and more than 30% are preventable. Additional causes like heredity and age are important unavoidable risk factors.[27,28]

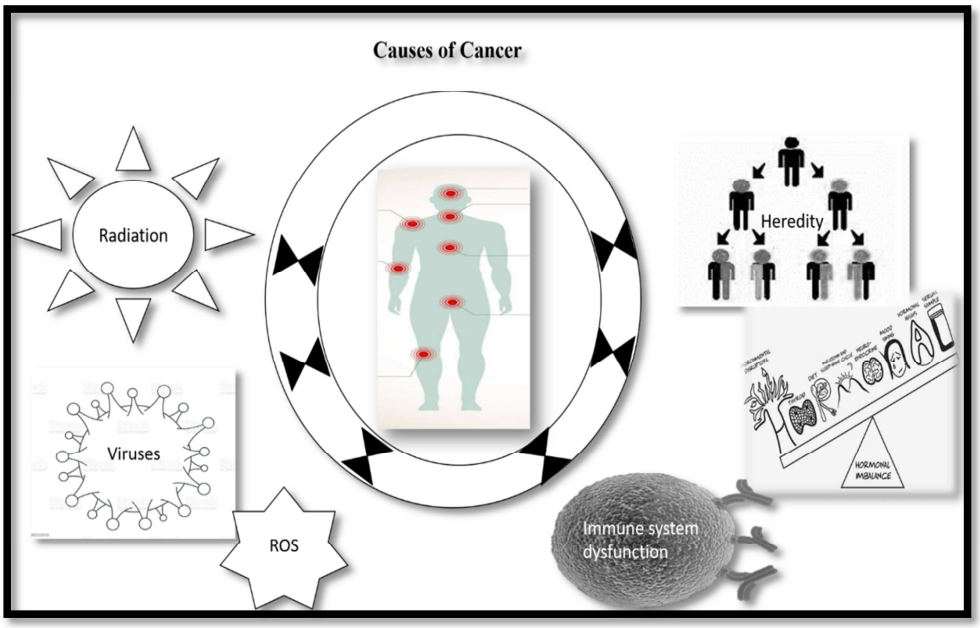


Figure 1: Highlight on causes of cancer

These factors lead to alterations in genetic material that regulate cellular functioning, particularly cell proliferation and cell division during multi-stage processes, which are typically the cause of the transformation of a precancerous stage into a malignant tumor. These genetic alterations may be inherited, result from errors during cell division, or be brought on by environmental influences (carcinogens).[29]

3.4. Management of cancer

Presently, numerous treatment modalities are available for cancer treatment. Depending on the type and stage of the disease, single or various combinations of drugs will be used to treat cancer. The most common approaches are explained briefly in Figure 2.

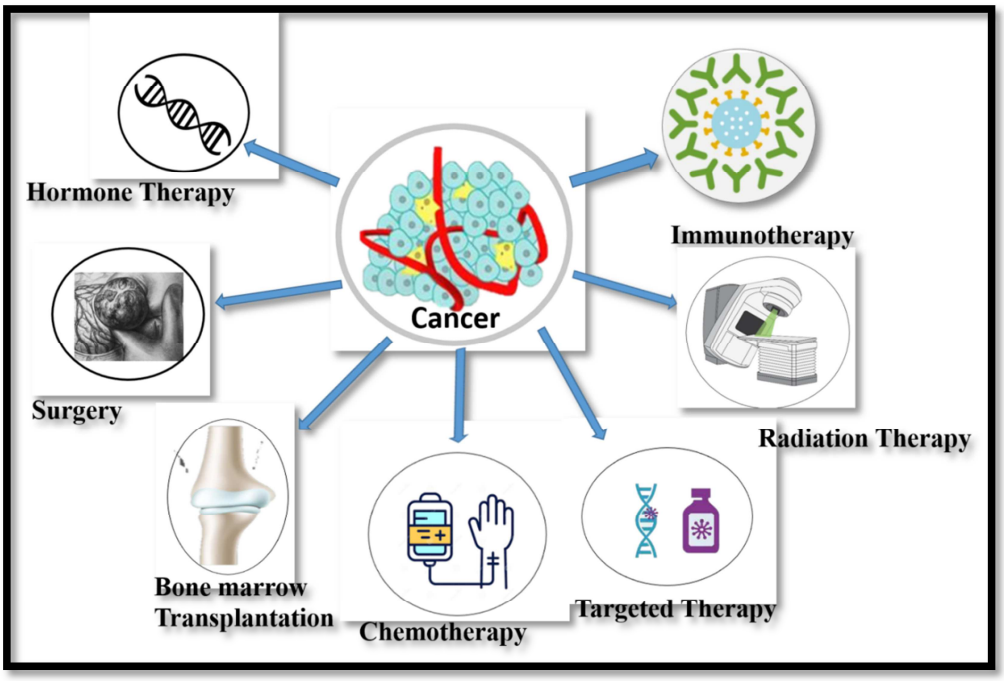


Figure 2: Highlight on Management of cancer

3.4.1. Surgery and radiation therapy [30]

The goal of the surgery is to remove tumor tissues without harming the rest of the body, but its effectiveness is frequently hindered by the invasion of surrounding tissues and the spread of microscopic metastatic cells to distant parts.

Radiotherapy (ionising radiation) is used to destroy cancer cells and causes the gradual shrinking of tumor size. However, it is suitable only when localised tumors confined to a specific region are only being treated. It damages the genetic material of

cells in the radiation-exposed area, making it impossible to grow and divide. Although most normal cells that divide slowly can recover radiation and bounce back to normal function slowly.[31]

3.4.2. Monoclonal antibody treatment and immunotherapy[32]

The term "cancer immunotherapy" describes a broad range of therapeutic approaches intended to make passive use of immune factors (antibodies, cytokines, etc.) or to increase the patient's immune system's capacity to fight cancer cells. With the finding of the structure of antibodies and the advancement of hybridoma technology, it emerged as a powerful technique that produced a reliable source of monoclonal antibodies (mAbs) against differentially expressed antigens on tumor cells, and these could be used effectively in the diagnosis and treatment of various cancers.[33–35] These will bind specifically to targeted receptors on the target cells and can be effectively applied in therapy in multiple ways, such as by inhibiting the multiplication and killing of cancer cells by blocking the specific cell receptors or key proteins in cancer cells. These mAbs are also effectively using radio-immunotherapy to localise the radioisotopes to the tumor microenvironment, which also assists in the delivery of lethal chemicals to the target specific cancer cells.[36] These developments made it possible to target tumors *in vitro* and *in vivo.*

3.4.3. Chemotherapy [37]

Chemotherapy is one of the most commonly used therapies for most cancers, which uses chemical entities to treat cancer. These are mainly targeted to inhibit or destroy the highly proliferative cells, which are the major hallmark of cancer.[38] Most classes of chemotherapeutic drugs used in clinical settings belong to systemic anti-proliferative agents targeting DNA and inhibiting the replication process in rapidly

multiplying cells.[39] Most of these drugs presently used in clinics are identified from natural sources like plants (vinblastine, vincristine), and microbes (Apratoxin A/Peptide, Sansalvamide A/Peptide). While some are developed synthetically (5-fluorouracil, methotrexate.) or through semi-synthetic modifications (irinotecan). Based on the target and mechanism of action, these agents are further grouped into such categories as DNA-interacting drugs (Cisplatin), anti-metabolites (Methotrexate), antimicrotubule agents (paclitaxel), and topoisomerase inhibitors (irinotecan, Doxorubicin). Although this medication has a high rate of killing malignant (tumor) cells, its efficacy is constrained by its harm to normally proliferating cells, majorly in the gastrointestinal tract, bone marrow, and cardio-vascular system due to its narrow therapeutic index and wide range of toxicities in various organs and organ systems.[40]

3.4.4. Targeted therapies at the molecular level

In the past few decades, insight into the molecular biology of cancer has improved, which has helped in the discovery and design of anti-cancer medications from conventional cytostatic or cytolytic to more focused and rationally designed specific targeted therapies, which greatly control the abnormal cell cycle, invasion, neovascularization, angiogenesis, and metastasis beyond the disruption of DNA replication.[41] Further, the identification of abnormal activation of signalling pathways which are directly linked to oncogenic processes leads to the discovery of general molecular targeted therapies that directly or indirectly affect several cellular processes involved in the progression of cancer. The receptor tyrosine kinase (RTK) signal transduction pathway is one of the earliest avenues of help in understanding the deregulation of the cellular networks in human cancers. The continuous activation of

these pathways by activating mutations in major proteins like epithelial growth factor receptor (EGFR) and Ras results in constant stimulation of these pathways and their involvement in cancer progression. Currently, the majority of the chemo and targeted therapies (mAbs-based or small molecules) are the RTK Inhibitors that target EGFR and other RTKs associated with cancer signalling pathways.[42]

3.4.5. DNA intercalating agents

Many DNA replication inhibitors like cisplatin covalently bind alkyl groups present in macromolecules like nucleic acids and proteins. These agents cross-link the adjacent bases and disrupt the structure and function, which leads to inhibition of replication, which in many instances causes mispairing, and consequently activates the induction of cell death pathways.[43]

3.4.6. Agents that interact with topoisomerase

The topoisomerases are the group of enzymes that play a vital role in replication, transcription, maintenance of chromosomal structure and function, DNA repairing mechanisms, and recombination processes, and these are most active in rapidly multiplying cells. Topoisomerase inhibitors are the group of chemicals that interfere with enzyme activity and prevent the re-ligation, thus stabilising the enzyme-DNA topoisomerase complex. Thus, by inhibiting DNA metabolism, affected cells are killed, activating apoptotic pathways. For example, doxorubicin (anthracycline class of topoisomerase II inhibitor) is the most commonly used chemotherapeutic in clinical practice for a variety of cancers.

3.4.6.1. Doxorubicin

In the 1960s, the chemotherapeutic medication doxorubicin was initially obtained from the bacterium *Streptomyces peucetius*. This drug is used to treat several cancers, including breast, stomach, myeloma, lung, and thyroid cancers.[44] It is generally documented that dox can intercalate into DNA and also block the enzyme topoisomerase II. It interferes with replication, transcription, and repair processes.[45] Dox, the most commonly used chemotherapeutic medication for triple-negative breast cancer (TNBC), promotes endothelial cell death in the tumor microvasculature when administered chronically and shows antiangiogenic properties.[46]

Additionally, dox therapy in malignancy can signal the damage-associated molecular pattern (DAMP) that can activate the body's natural defences against tumors. The only disadvantage of dox therapy is that it is toxic to normal fast-growing cells.[47–50] According to Poljakova,[49] cytotoxic medicines affect cells that develop quickly by stopping the growth cycle. Some normal cells, like which, develop more quickly, such as hair follicles. As a result, dox kills both cancer and healthy cells. Dox at higher doses is quite cytotoxic and causes some adverse effects. Hair loss, nausea, vomiting, mouth sores, darkened or blackened urine, diarrhoea, bloody stools, red patches or bruises on the skin, shortness of breath, abdominal pain, cardiotoxicity, hepatotoxicity, and nephrotoxicity are some of the more common adverse effects of dox. ADR is dose-dependent and gets worse with dosage.[51] Initial retrospective investigations showed that doxorubicin toxicity and heart failure were dose-compared, with the incidence of problems significantly rising when the total dose was greater than 550 mg/m^2 of body surface area. When the cumulative dose was between 500 - 550 mg/m^2, the rate of heart failure was around 4%, but when the dose was increased

to between 551-600 mg/m^2, it increased to 18%, and when the total dose was at least 601 mg/m^2, it increased to up to 36%. [52]

As with other anti-cancer medications, doxorubicin has a double-edged nature in that it can increase tumor cell resistance while simultaneously being hazardous to healthy organs. They are proven to cause cardiomyopathy, which results in a type of chronic cardiac heart failure that is typically resistant to treatment with conventional drugs.[53–55]

In light of the side effects, the generation of free radicals, which causes oxidative stress and leads to oxidation-mediated damage across the various organs, is the most important mechanism of dox-induced toxicities.[56,57] Various studies explain the toxicities produced by doxorubicin in that some are summarized. Wang *et al.,* explained the negative effect of dox (20 mg/kg, i.p.) on mice's duodenal epithelium, which results in the release of gut microbiota compared to endotoxin, as a result of increased TLR4 signalling. This results in immunotoxicity through widespread inflammation and multiple organ damage.[58] Further, Reddy *et al.,* , showed significant degenerative alterations in the heart, liver, kidney, and testis.[59] Another study showed changes in biochemical indicators such as ALT, AST, TC, total cholesterol, and BUN after receiving dox therapy.[60] These reports help determine the vital-organ toxicity of dox.

Table 1: Toxicity of Doxorubicin is summarized

S NO.	Human cell line or cell culture /Animal Model	Drug Doxorubicin dose	Observed characteristics/ Organ toxicity	Reference
1	3D Microfluidic system of serum-free medium with different cell types models.	5 μM	Liver, cardiac, neuronal, and muscle toxicity	61
2	Model of mice species C57BL/6N	Dose given 20 mg/kg, Route: i.p.	Observed Systemic inflammation, Organ-specific inflammation.	58
3	Male albino Wistar Kyoto rats	Dose given mg/kg, Route: i.p.	Congestion and degenerative in multiple organs like changes in the heart, liver, kidney and testis.	59
4	Male Sprague Dawley rats	Dose given 20 mg/kg, Route: i.p	Observe the change in liver and kidney biochemical parameters.	60
5	Cardiomyocytes from pluripotent derived stem cells.	The dose is given 450 nM	Observed the upregulation of TNFR1, FAS, DR4 and DR5 receptors and decrease in cell viability	62
6	ICR outbred model mice albino strain	Dose given 20 mg/kg, Route: i.p.	Change in different Biochemical parameters	63
7	Wistar albino rats	Dose:2.5 mg/kg, Route given i.p.	The observed change in serum cardiac and Biomarkers of lipid, Change in ECG. (Electrocardiogram)	64
8	Male Wistar rats	Dose given 25 mg/kg Route: i.p.	Noted swelling, Hydropic degeneration of tissue,	65
9	Male adult Swiss albino rats	Dose:7.5 mg/kg Route given I.V.	Change in renal biochemical profile.	66
10	Male Balb /c mice	Dose given 5mg/kg i.p. twice/week for the duration of 2 weeks	Increases in apoptotic cell death and inflammatory markers.	67

11	Male BALB/c mice	Dose given 11.5 mg/kg	Increases in apoptosis, inflammation, and oxidative stress (ROA)	68
12	Mice model	2.5 mg/kg, i.p.	Increased plasma ALT and AST levels.	69
13	Female Wistar rats	Dose given 1.8 mg/kg, Route given i.p.	Decreased level of malondialdehyde (elevated) and glutathione peroxidase in liver	70
14	Male SD rats	Dose given 15 mg/kg. For the duration 2 weeks	The observed rise in MDA and decrease in SOD levels in the brain	71

3.4.6.2. Mechanisms of Cardiotoxicity Caused by Dox

Numerous early attempts were made to understand the dox-induced cardiomyopathies and the pathways involved, however, it is still unclear. There is some evidence which suggested that dox-mediated multi-organ toxicities are linked to oxidative stress due to ROS, inflammation (Inflammatory markers), and apoptosis (Programmed cell death).[72]

Cardiotoxicity is considered an extremely serious side effect of doxorubicin during cancer treatment. Even preclinical studies proved cardiotoxicity during dox treatment. According to Mandziuk *et al.*, treatment with dox caused changes in histology and biochemical markers such as cardiac superoxide dismutase, brain natriuretic peptide (BNP) and fatty acid-binding protein. In contrast to this, some of the co-administered medications can reduce the accumulation of dox in the organ.[73]

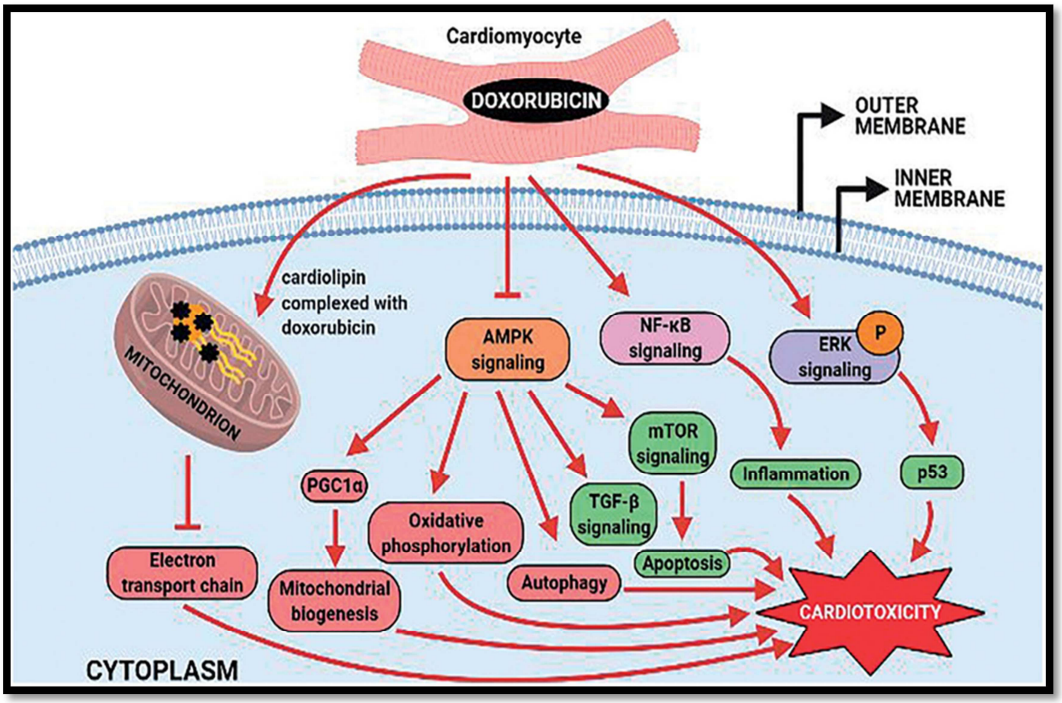


Figure 3: The mechanism involved in dox-induced cardiotoxicities was explained.

Figure 3: Cardiac tissue, the most severely impacted by doxorubicin, activates and inactivates signalling pathways. Positively charged Dox binds to cardiolipin, blocking oxidative phosphorylation. Inhibition of AMPK signalling alters pro- and anti-survival factors, causing myocardial apoptosis. Activating the NF-jB signalling pathway causes inflammation, and phosphorylating the ERK pathway activates p53. Both procedures kill heart tissue. AMPK inhibits the mTOR pathway that activates apoptosis. Source[74]

3.4.6.3. Mechanisms of Hepatotoxicity Caused by Dox

Doxorubicin-mediated hepatic changes are well-reported both clinically and preclinically.[75] Kocahan *et al.,* and Zhao *et al.,* investigated the alteration of the liver parameters, the significantly elevated levels of alanine aminotransferase, aspartate

transaminase, and malondialdehyde and reduced levels of glutathione peroxidase were reported during dox treatment. The generation of ROS, which causes an imbalanced cellular redox potential which leads to hepatotoxicity, is the primary cause of doxorubicin-induced oxidative stress. [69,70]

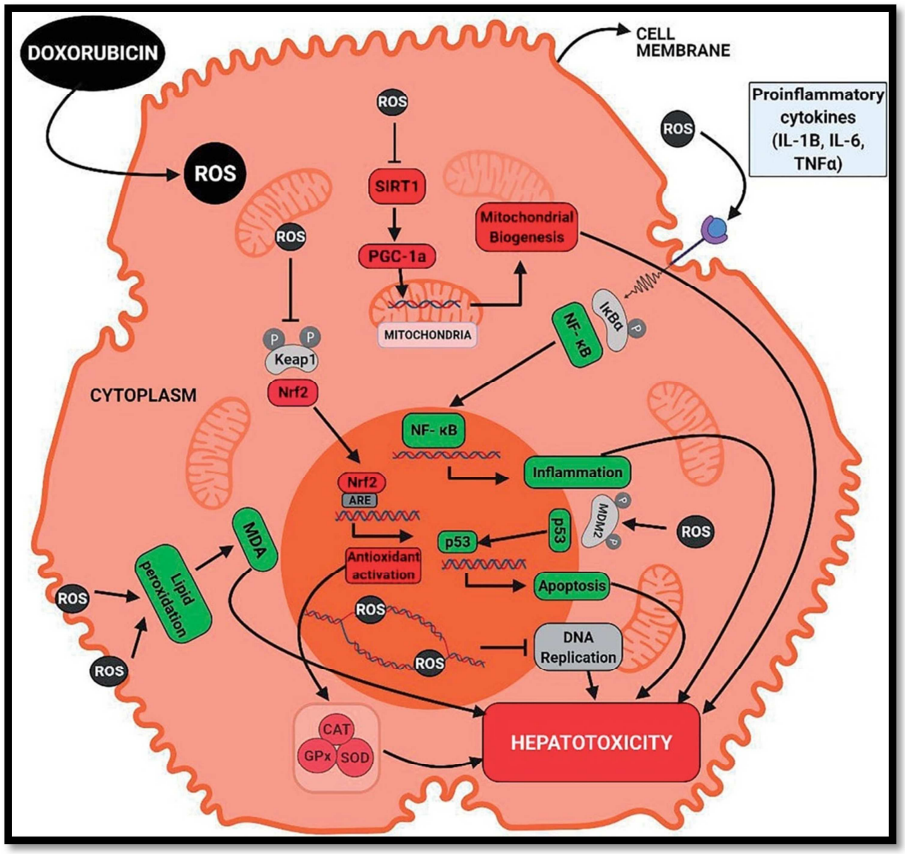


Figure 4: explains the doxorubicin-induced hepatotoxicity process.

Figure 4: Activation of many pathways, including the inflammatory process via NF-jB signalling, inactivation of antioxidative genes, and mitochondrial biogenesis produce doxorubicin-induced hepatotoxicity. ROS inhibits Nrf2 and keap1 phosphorylation. It promotes MDM2 and IjBa phosphorylation, activating NF-jB and p53. Active p53 and NF-jB induce liver apoptosis and inflammation. Liver injury causes all of these.[74]

3.4.6.4. Urinary system effects

A multi-organ impairment that is largely made possible by the formation of free radicals and finally results in the membrane lipid peroxidation may include dox-impelled renal toxicity because it accumulates particularly in the kidney, dox can promote nephrotoxicity which is evidenced by increased glomerular capillary permeability, and tubular degeneration.[76,77] Djabir *et al*, Showed that oedema, vascular degeneration, vacuolization, pyknotic cells and necrotic alterations were visible in renal tissue. Further, El-Sayed *et al.*, also discovered that lower levels of GSH and SOD were associated with higher levels of creatinine, urea, TNF alpha, cyclooxygenase-2, caspase-3, malondialdehyde, and nitric oxide activity.

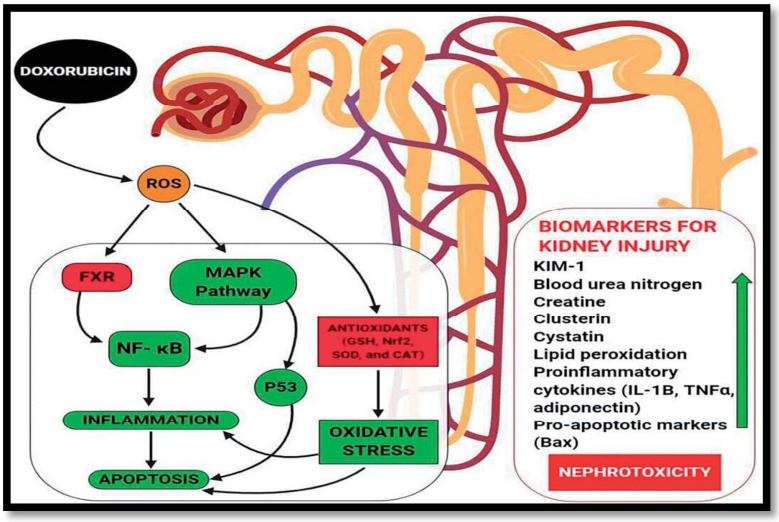


Figure 5: Explains the doxorubicin-induced nephrotoxicity process.

Figure 5: Doxorubicin caused oxidative stress and nephrotoxicity. It activates the signalling pathway NF-jB, which releases inflammatory cytokines like IL-1b and TNF-a, causing kidney inflammation. Downregulated FXR activates the NF-jB signalling pathway. All events eventually start the apoptotic process with the help of Bax.[74]

3.4.6.5. The mechanism for organ protective effect of specific drugs against toxicity caused by dox

Contrary to dox-induced toxicity, antioxidant-rich substances demonstrated a preventive role against dox-induced organ toxicity.[78] Reduced cardiac apoptosis and thus basis for cardioprotective effect.[79] According to Zhang *et al*., Tannic acid can prevent oxidative stress, inflammation, and apoptotic damage suggests that it may be able to prevent the cardiotoxicity caused by dox.[72] Anthor study, where freeze-dried strawberry fruit powders may be able to treat illnesses linked to oxidative stress by reducing the oxidative damage in cardiotoxic groups in rats due to the active compounds present in strawberry fruits.[80] The reduction of intracellular ROS produced from dox on Vero cells and myocyte cell H9c2(2-1) was caused by the methanol extracts of *Averrhoa bilimbi'* (BM) *in vitro* antioxidant activity. As a result, the cardioprotective benefits of some drugs against dox-induced cardiotoxicity may be significantly influenced by ROS inhibition.

Different reported studies have shown that dox administration can affect the hepatic system. Mete *et al.,* have shown that *Allium cepa* plant extract protects against dox-induced hepatotoxicity in rat model.[75] Naringenin shows the potential impact on dox-induced hepatotoxicity, likely through reducing oxidative stress and inflammation, which are closely compared to dox-triggered damage.[81] Further, *Kleinhoviahospita* L extract. and *Phoenix dactylifera* extract has been reported to reduce liver toxicity and nephrotoxicity induced by dox due to its antioxidant properties.[65,82] The tannin-rich ethyl acetate fraction of Acacia hydaspica may have nephroprotective effects due to its active metabolite's antioxidant and free radical-scavenging properties.[83]

3.4.6.6. Combination therapies

Anthracyclines are extremely effective anti-tumor drugs; thus, their widespread use will continue until safer and more suitable substitutes are found. Based on an apparent ability to adapt some of the biochemical abnormalities that result from doxorubicin administration, a wide range of substances are tested in various animal models for their potential to reduce doxorubicin cardiotoxicity. Through a caspase inhibitory activity-dependent mechanism, the combined effects of Adriamycin and amphotericin B result in a significantly higher cell death index of the MCF-7 cell line (breast cancer).[84] As per the studies, the concomitant administration of certain drugs with dox can increase their anti-cancer efficacy while reducing their hazardous side effects.

3.5. *Theobroma cacao* L.

3.5.1. Taxonomy Classification[85]

Kingdom: Plantae

Division: Tracheophyta

Class: Magnoliopsida

Order: Malvales

Genus: Theobroma L.

Species: *Theobroma cacao* L.

Botanical name: *Theobroma cacao* Linn.

Family: Malvaceae

Parts used: beans

3.5.2. Origin and distribution

Tropical evergreen cocoa trees are perennial diploid (2n=20) plant species grown for their edible cocoa beans, which are used to make cocoa butter and powder. The plant was given the name "*Theobroma cacao*" by Swedish botanist Carolus Linnaeus in the 18th century (1737), which is now known as its official botanical name and translates to "food of the gods" in Greek.[86] Cacao tree, chocolate tree, and *Theobroma sativum* are a few of its common names. *Theobroma cacao* is a member of the angiosperm kingdom Plantae, belonging to the family Malvaceae. known as Sterculiaceae (sterculia), and the genus Theobroma. It spread to Central America, mainly Mexico. Theobroma, which is derived from the Greek terms broma and theo, means "food" and "of the gods," while cacao is a word derived from "Aztec Nahuatl term from the words xococ (bitter) and atl (water)". The production of cocoa is widespread today. Large-scale cocoa cultivation started in India in the 1970s, and now it is widely grown as an intercrop in Kerala, Tamil Nadu, Andhra Pradesh and Karnataka.[87]

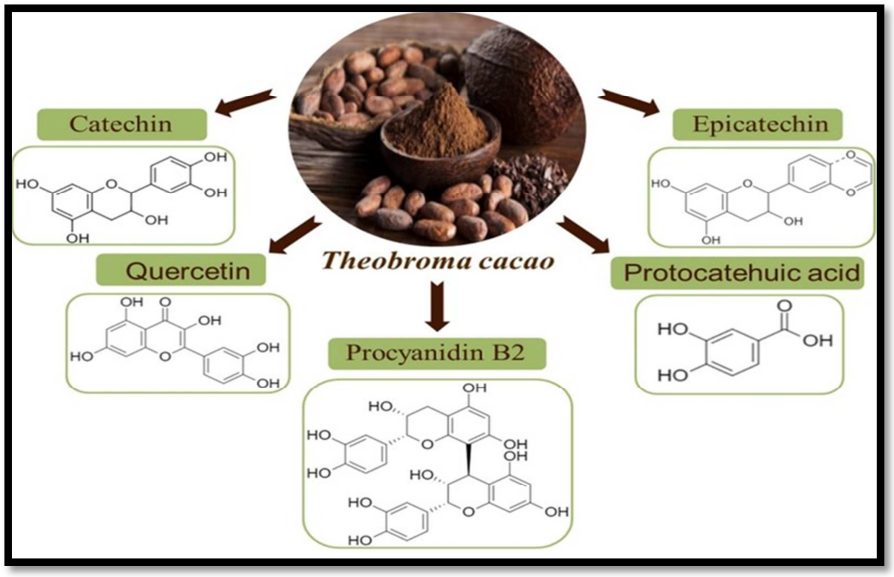


Figure 6: Major polyphenol found in cocoa

3.5.3. Traditional/ethnomedicinal uses

Ancient peoples employed cocoa as a medicinal herb to heal various ailments. From the 16th to early 20th century, over a hundred therapeutic uses of cocoa were recorded in Europe and New Spain; including the treatment of anaemia, fatigue ness, TB, fever, gout, kidney stones (Urolithiasis), and even a lack of libido.[88] There are different phytochemicals in the cocoa bean, many of which have been reported for various physiological effects;[89] stated that selected procyanidins found in cocoa reduce the chance of cancer. Colon cancer Caco-2 cells were subjected to cocoa seed extracts, which resulted in the arrest of 70% growth inhibition. Consuming cocoa or chocolate, which has an intense antioxidant activity, may help reduce the harm caused by epigenetic cancer and block the intricate processes resulting in cancer.

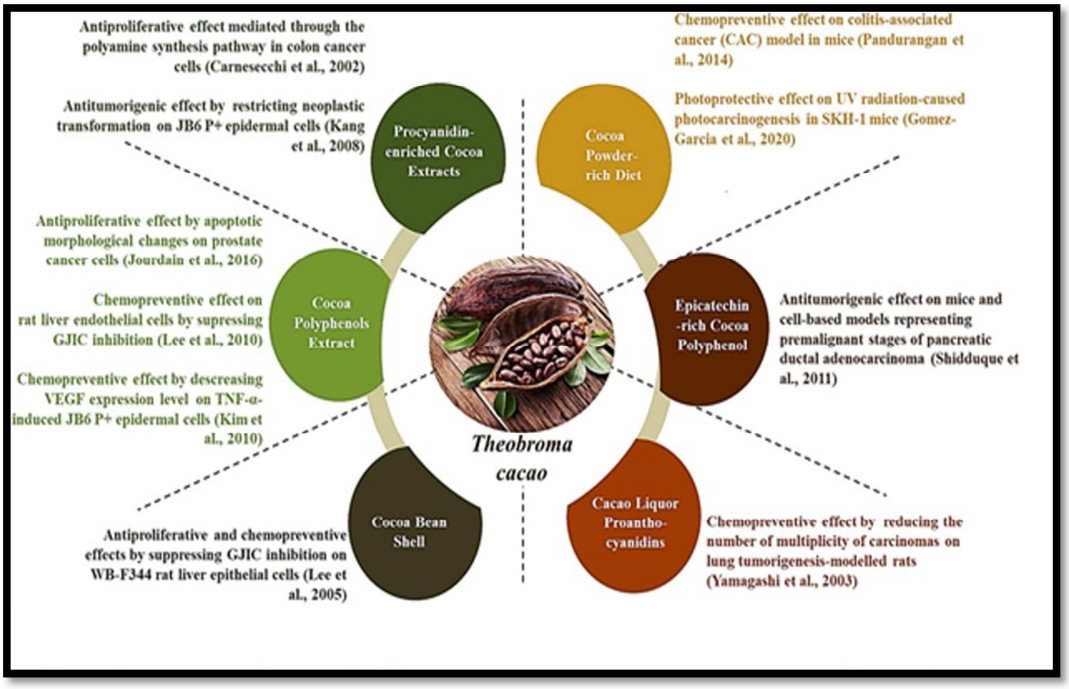


Figure 7: Summary of the antiproliferative, apoptotic, and chemo preventive effects of cocoa on different *in vitro* and *in vivo* cancer models

3.5.4. Pharmaceutical properties

Plants are rich sources of phytochemicals and minerals. It is consumed as a food commodity in the world.[90] The flavonoids in cocoa, which have antioxidant properties, aid in preventing diseases brought on by oxidative stress. MytomycinC produces reactive oxygen radicals that cocoa polyphenols scavenge, avoiding DNA deterioration.[91] Cocoa polyphenols protect against ROS and oxidative stress caused by hydrogen peroxide, which is linked to many diseases like DM (diabetes), AD (Alzheimer's), and other neurodegenerative disorders. Even in colon cancer, cocoa has demonstrated antiproliferative potential by lowering levels of ERK, Akt, and cyclin D1.[92]

Chemical mutagens are hazardous environmental carcinogens that alter DNA and may lead to cancer. Cacao exhibits antimutagenic properties by inhibiting the activation of cytochrome P- 450, specifically CYP1A and VEGF expression.[93] The phytochemicals in cocoa, particularly the flavonoids, are engaged in several processes and serve as molecular targets for the treatment of human cancer cells.

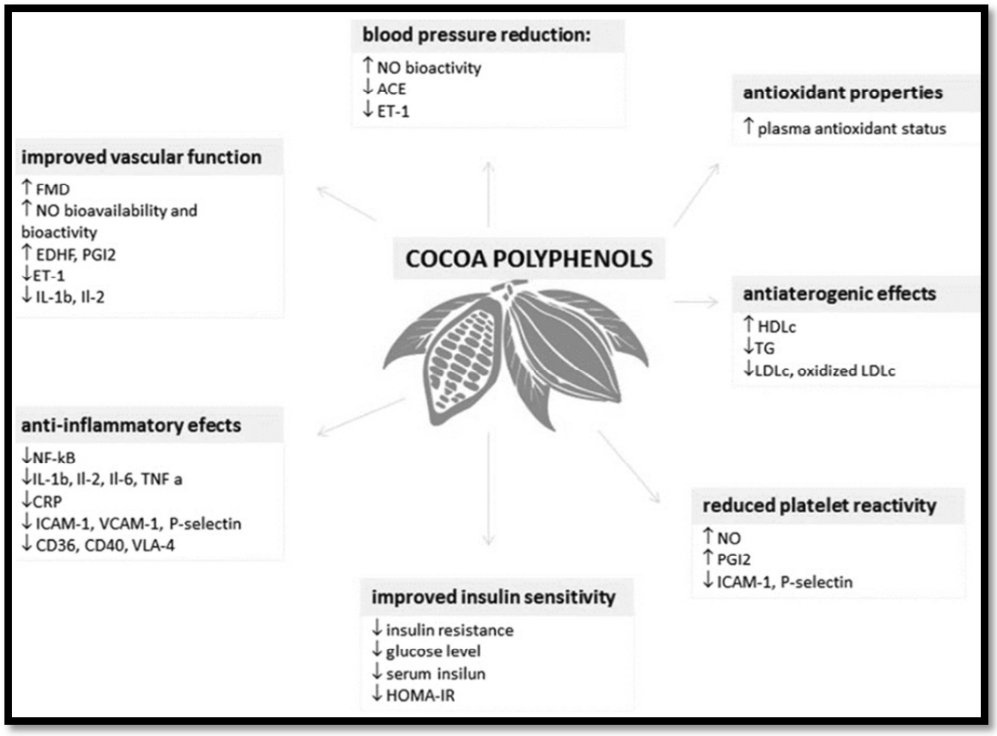


Figure 8: Cardiovascular disorders (CVS) can be prevented because of the therapeutic effects of cocoa components. By creating a positive effect on platelet aggregation, lowering blood pressure, lowering the level of dyslipidemia, and lowering blood sugar, it lowers the risk of cardiovascular disease. Numerous research has demonstrated the anti-inflammatory, antioxidant, and important function that cocoa flavanols play in reducing insulin resistance.

3.5.5. Cocoa on organ protective activity

Diet plays role in lifestyle factors that can knowingly affect different diseases. In addition to having an anti-inflammatory impact, cocoa polyphenols also aid in the body's elimination of dangerous free radicals, which prevents the development of numerous chronic diseases such as tumor, diabetes, CCF, liver disease, and renal disease.[12,26,94]

As per almoosawi *et al.*, , a clinical trial revealed that consuming polyphenol-rich chocolate for 2 weeks had an effect on the BP levels of obese people their study revealed alterations in systolic and diastolic blood pressure, fasting blood glucose levels, and urinary-free cortisone levels as well as in preclinical study showed the rats' which received cocoa leaf and bark extract showed protective effects against dox-induced oxidative stress and organ toxicity (splenotoxicity, cardiotoxicity, and nephrotoxicity).[22,23,95] Healthy young people's blood oxygen level-dependent response to memory activities switching paradigm is increased by flavanol-rich cocoa[96] It might be helpful in the treatment of stroke, Alzheimer's disease, and cerebrovascular flow (CBF) dementia.[97,98 99]

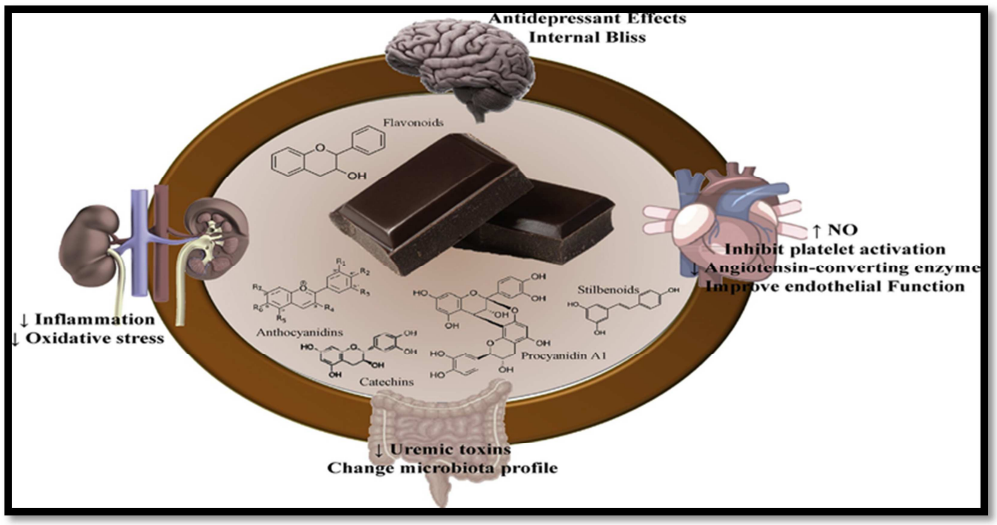


Figure 9: Possible effects of chocolate. The polyphenol in cocoa may lessen renal inflammation, intestinal microbiota-produced uremic toxins, and kidney oxidative stress. Similarly, these substances may act as an antidepressant and have numerous positive effects on the cardiovascular system (CVS), raising levels of (Nitric oxide) NO and enhancing endothelial function.

3.5.6. Cocoa protective activity on heart

There are several processes by which cocoa flavonoids protect against cardiovascular disease. These include enhancing endothelial function, lowering blood pressure, exhibiting antioxidant, antiplatelet, and anti-inflammatory properties, as well as potentially altering lipid patterning (These effects may reduce cardiovascular risk). In ancient times Indian tribes like kuna and martens use cocoa to treat blood pressure.[100] Martens had reduced blood pressure and also no age-compared kidney deterioration, according to clinical studies.[101] In addition, local population deaths from CVS events were knowingly reduced than other americans.[102] Now homemade cocoa is replaced with other low-flavanol foods. A cross-sectional investigation found that consumption of cocoa was inversely connected to blood pressure and was also linked to lower rates of cardiovascular and all-cause mortality.[103]Several potential mechanisms have been proposed by which cocoa and flavanols can improve cardiovascular health

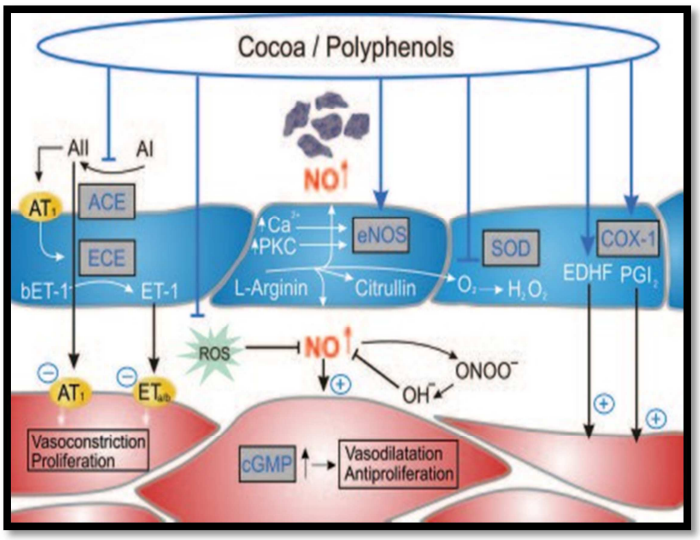


Figure 10: Endothelial cells with cocoa polyphenols. Endothelial cells emit NO in reaction to shear stress from acetylcholine, 5HT. eNOS creates NO from L-arginine in

tetrahydrobiopterin. It increases Calcium+ or phosphorylates eNOS through PI3-kinase/Akt. *In vitro,* it reduces vascular arginase action in human endothelial cells, raising local L-arginine levels. NO releases cGMP, which regulates smooth-muscle cells. NO promotes vasodilation and inhibits platelet and leukocyte adhesion, migration, and proliferation. NO-mediated mechanisms are discussed. Antioxidants can improve endothelial function by reducing reactive oxidative radicals. Cocoa polyphenols reduce endothelin-1 (ET) production, increase endothelial prostacyclin production, and activate EDHF (EDHF). Polyphenols can block angiotensin-converting enzyme.[104]

3.5.7. Cocoa protective activity on liver

Liver steatosis is a condition in which the level of lipids in the liver is greater than five per cent; it results in nonalcoholic steatohepatitis. NASH and steatosis are reversible, but they can lead to permanent liver fibrosis and, ultimately, cirrhosis and hepatocellular carcinoma.[105] People with NAFLD who consume 40 g of dark chocolate per day have significantly lower plasma concentrations of 8-isoprostane, ALT and (NOX)-2 (soluble NADPH oxidase-derived peptide).[106] Janevski *et al.,* reported that the addition of a high-fat/low-methionine diet to SD rats containing 125 mg/g of cocoa powder reduced steatosis and hepatic inflammation.[107] Alavinejad *et al.,* conducted a study involving 42 patients and found that cocoa, in the form of dark chocolate, reduced harmful enzymes linked to liver damage. The cocoa polyphenol extract inhibits apoptosis and increases antioxidant capacity.[108] There is ample evidence that the live-protective effects are due to a phytochemical in cocoa: polyphenols. These compounds have antioxidant activity but also other biological effects.

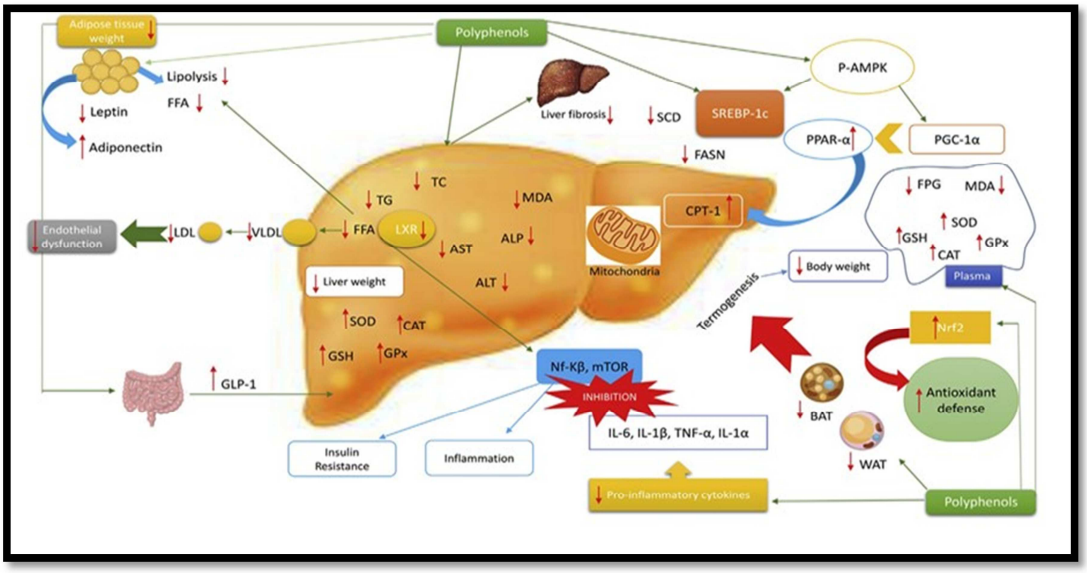


Figure 11: Possible mechanism of polyphenols effects on NAFLD according to preclinical studies

3.5.8. Cocoa protective activity on kidney

Preclinical studies in mice have demonstrated renoprotective activity for various (poly) phenols derived from cocoa, including flavanols. They showed that nutraceuticals could lessen oxidative and inflammatory stress, renal damage, and the onset of renal failure.[109,110] According to research in spontaneously hypertensive rats with diabetes induced by streptozotocin, the activation of polyphenol-enriched cocoa-enriched AMPK could inhibit the TGF-β1/Smad signalling pathway by blocking TGF-1, collagen four and fibronectin.[110]

3.6. Experimental models for anti-cancer activity

The potential medicine goes through several qualifying investigations before any potential anticancer agent is tested on humans. First, a potential candidate's capacity to prevent the growth of tumor cells in culture is assessed through primary screening utilising the human tumor cell line anticancer drug screening technique.

Previous reviews of several cells and tissue culture platforms for anticancer drug screening were in-depth.[111] The potential medicine then undergoes *in vivo* animal experimentation following the preliminary *in vitro* assay. Inbred laboratory mice are used for the vast majority of *in vivo* cancer research.[112] Mouse tumor models are used for a variety of reasons, including the similarity of the human and mouse genomes, the affordability of housing and upkeep, the short gestation period and high rate of reproduction, and the rapid growth rate of implanted tumors. An inbred laboratory mouse is a great tool for studying cancer and screening prospective anticancer drugs due to these and other factors.

3.6.1. Human tumor mouse xenotransplanted models

In 1969, immune-deficient mice were successfully implanted with *in vitro*-cultured human tumor cells.[113] The malignancy is not rejected because the typical adaptive immune responses related to the rejection of foreign substances, such as killer T cells that cause the death of target cells and humoral immune responses mediated by activated B cells secreting antibodies, are suppressed. An anticancerous treatment is introduced when the tumor reaches a specific size, and the medicine's efficacy is determined by variations in the tumor size. For the simplest xenotransplantation paradigm, tumor cells are subcutaneously injected. This method makes it possible to quickly calculate a compound's toxicity and antitumor activities. [114] The model's attractiveness is that an animal's surface is covered in a tumor, making it simple to measure the tumor volume and track the progression of the disease.[115] To predict the clinical effects of medications like cyclophosphamide, whose action is not tumor-dependent, subcutaneous xenotransplants have been employed successfully.[116] In subcutaneous xenotransplanted rats, these substances were shown to be successful

in treating rhabdomyosarcoma and adenocarcinoma of the human colon. Finally, extra attention should be given to the differences between people and mice when determining the dose of a potential drug under study. The majority of chemotherapeutic medication doses needed in mice to provide a suitable clinical effect are typically 4-5 times higher than those needed in humans.[117,118] As a result, the dosage of the drug necessary to provide a clinically important benefit in humans is occasionally insufficient to find a significant effect in mice but research has shown that a clinically relevant dose in rodents for tumors growing as mouse xenotransplants is frequently equivalent to that observed for human primary cancers.[119]

3. MATERIAL AND METHODS

To examine cocoa's organ-protecting and anti-cancer properties either alone or in conjunction with doxorubicin, an *In silico*, *in vitro*, and *in vivo* pharmacological methodology was applied.

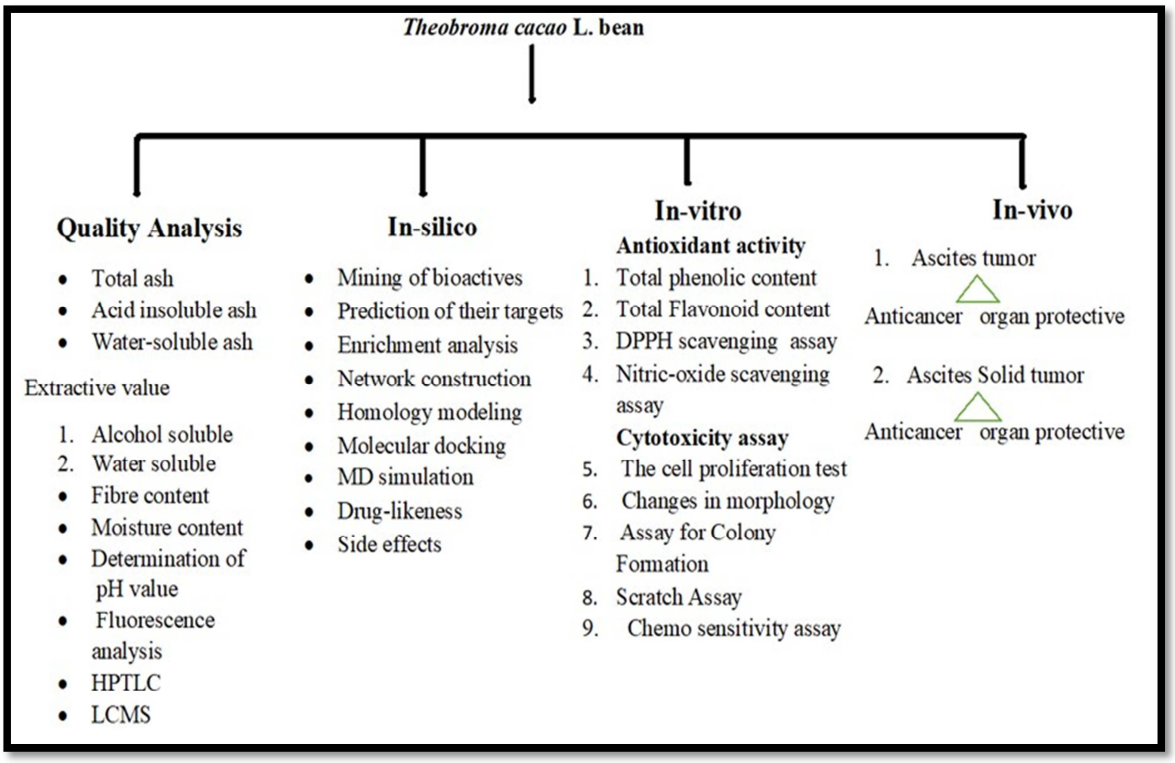


Figure 12: Schematic representation

3.1. Collection of plant material and authentication

Cocoa nibs were harvested from the local area of Sirsi India, (14°.34'38.7984 N, 74°.58'21.288 E); authenticated by plant taxonomists from ICMR-NITM Belagavi and deposited the herbarium in ICMR-NITM. (Voucher number RMRC-1392).

3.2. Physiochemical Analysis[120,121]

Physical-chemical analyses of plant products, like total ash, water-soluble ash, alcohol soluble ash, water-soluble extract, acid-insoluble ash, and raw fibre content in cocoa raw materials, were performed to ensure that they were of high quality as per Ayurveda Pharmacopoeia. (1999)

3.2.1. Ash Values

The ash value shows the presence of several impurities, such as carbonates, oxalates, and silicates, and it is utilized to assess the purity and quality of the raw material. Silica makes up the majority of the soil-contaminated acid-insoluble ash, which also contains other minerals. To determine how many inorganic elements are present in plant materials. The Ayurvedic Pharmacopoeia protocol was used in this work to analyze the acid-insoluble ash, water-soluble ash, and total ash in crude cocoa. (1999)

3.2.2. Total Ash

The coarse and dried form test material was weighed for 2 g in a silica crucible and placed in the incinerator at 500℃ till it converts into ash. The crucible was weighed again after cooling and the total ash content (%) was calculated.

$$Total\ ash\ = Ash\ weight\ /initial\ weight\ of\ powder\ (g)\ \times 100$$

3.2.3. Water-soluble Ash

Ash was heated in 25mL of water first, then filtered through ash-free filter paper (Whatman 4.1), then Whatman paper was rinsed with warm water, followed by dried, and burned in a silica container. The ash that is insoluble in water was weighed

after cooling. When calculating, the total ash was subtracted from the water-insoluble ash.

$$Water\ soluble\ ash\ = water\ soluble\ ash\ weight\ /initial\ weight\ of\ powder\ (g)\ \times 100$$

3.2.4. Acid-insoluble Ash

Using 45 ml of dilute HCl, the ash obtained from the total ash was boiled for 5 minutes. The mixture was filtered through ash-free filter paper, and the resulting residue was washed with hot water. The filter paper has been ignited and the weight was recorded after it had completely dried. The acid-insoluble ash (%) was determined.

$$Acid\ insoluble\ ash\ = Acid\ insoluble\ ash\ weight\ /initial\ weight\ of\ powder\ (g)\ \times 100$$

3.2.5. Extractive Value

The plant's materials consist of several metabolites which are responsible for their pharmacological activities in the form of primary and secondary metabolites. These metabolites can be extracted with different solvents using various extraction procedures. The number of bioactive components retrieved from a specific quantity of plant material using various solvents is known as extractive value.

3.2.6. Water Soluble Extractive

The 4 g of coarse powder of cocoa was macerated with 100 ml of water in 250 ml of a conical flask for 24 h. After shaking frequently for 6 h the flask was allowed to stand for 18 h. It was then filtered and 25 mL of the filtrate was evaporated to dryness in a flat-bottomed flask, dried at 105 ± 1°C and weighed. The percentage of water-soluble extractives was calculated.

3.2.7. Alcohol Soluble Extractive

4 grams of coarsely powdered cacao were macerated in 100 mL of the specified strength of alcohol for 6 hours, and then the mixture was allowed to stand for 18 hours in a closed flask. To avoid alcohol loss, the mixture was promptly filtered. 25 mL of the filtrate was then dried (105°C) and weight was calculated. The extractive % that is solvable in alcohol was calculated using air-dried cocoa.

3.2.8. Crude fibre content

Powered cacao was properly weighed and added to a porcelain dish at a rate of around 2 g. 10 % Nitric acid solution of around 50 mL was added, heated for 30 seconds while being constantly stirred, and then filtered through a fine-mesh cotton cloth. 5mL of hot water was used to wash away the residue. The cloth's scraps were collected in a porcelain dish and added 50 mL of 25% caustic soda was. After that, cotton cloth with a fine mesh was used to filter the liquid. 100 mL of hot water was used to rinse the residue. After filtering, the fibre was collected in filter paper, dried at 105 ℃, and weighed. The crude fibre content was calculated using the residue's weight.

$$Crude\ Fibre\ Content = weight\ loss/sample\ weight \times 100$$

3.2.9. Moisture content

The estimation of moisture content is a % loss in weight (w/w). Crude materials containing water or volatile matter can be measured by this method. Initially, a glass-stopper bottle weight was noted. Accurately weighed 2 gm of the test material was placed in the bottle, again the weight of the bottle and test material was noted. The bottle loaded with test material was placed into the oven. The material was

dried till it get constant weight readings. After cooling the bottle final weight was noted to determine the moisture content (%).

$$Moisture\ content\ (\%)\ = Loss\ in\ weight/weight\ of\ powder\ (g) \times 100$$

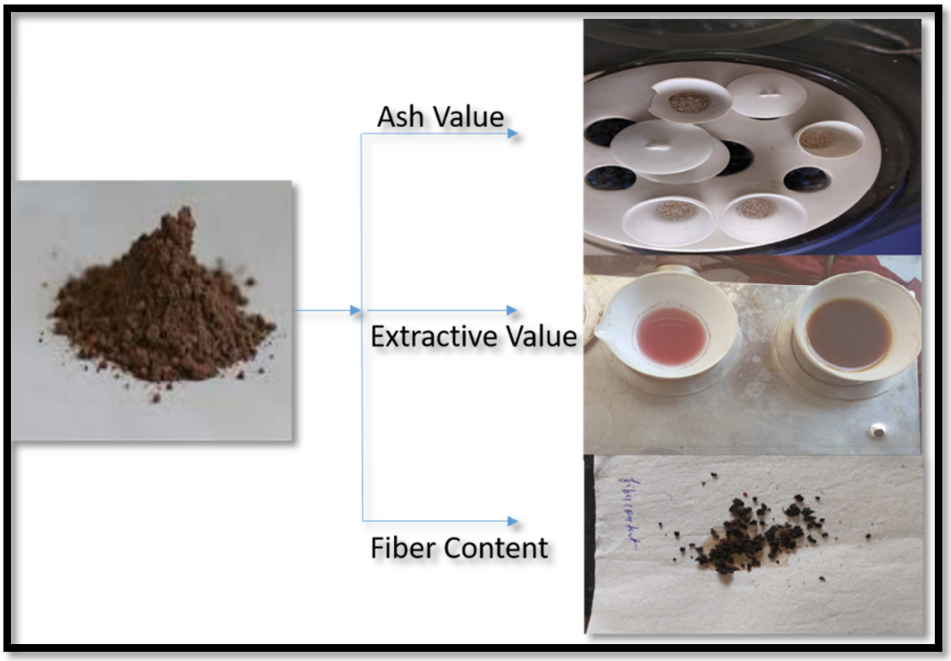


Figure:13 Physiochemical Analysis

3.2.10. Determination of pH value

Using a glass electrode and an appropriate pH metre, the pH of the 1 per cent w/v filtered cocoa solution was calculated potentiometrically.

3.2.11. Fluorescence analysis of powder cocoa.

By combining the cocoa powder with several reagents such as HCl, H_2SO_4, HNO_3, $FeCl_3$, and picric acid, which can be observed in a UV chamber at 254nm, 366nm, and visible light, fluorescence examination of the mixture was performed.

Table 2: Preliminary Phytochemical Analysis of the Extracts

Test	Method	Observation
Tannins	Ferric chloride test Dilute extract + 2mL ferric chloride solution	Dark blue colour
Alkaloids	Dragendorff's test Extract + Dragendorff's reagent	Yellow/ orange precipitate
Phenols	Ferric chloride test Extract +H2O2+ $FeCl_3$	blue colour
Glycosides	Molisch's Test Extract + α-naphthol + 1mL Conc. H_2SO_4	Violet ring at the junction of two layers
Reducing sugars	Fehling solution test Extract + Fehling solution A & B- boiling in a water bath	Brick red precipitate
Amino acids	Ninhydrin test Extract hydrolysis with HCl + 0.1% Ninhydrine solution	Violet purple colour
Flavonoids	Shinoda test Extract + Mg Turnings + 2 drops of Conc. HCl	Pink colour

3.3. In silico

3.3.1. Bioactive mining and target prediction

The bioactives from cocoa were listed from the literature and their structures in "smile" and "sdf" file format were then retrieved using publicly available small molecule databases like phytochemical interactions database(PCIDB; https://www.genome.jp/db/pcidb/), Dr. Dukes DB (https://phytochem.nal.usda.gov/phytochem/search). The targets were predicted using Binding DB correspondence to

the known ligand molecules having minimum similarity of >0.7 and their Gene IDs were retrieved from UniProtKB (https://www.uniprot.org) database.

3.3.2. Pathway and network construction

A set of Gene IDs was submitted to the search tool for the Retrieval of Interacting Genes/Proteins (STRING; https://string-db.org/)[122] 11.0v to identify the protein-protein interaction and pathways modulated by the predicted targets. The overall pathways modulated by the gene set were identified using the KEGG pathway (https://www.genome.jp/kegg/). The network between compounds, targets, and pathways was constructed using Cytoscape[123] ver 3.6.1. Biological interactions among them were interpreted using an “edge count” The map node size was set from ’low values to small sizes’ and the map node color from ’’low value to bright colors" was set for the network.

3.3.3. Epidermal Growth Factor Receptor (EGFR) structure refinement and active sites assessment.

EGFR is a potential cancer target and a highly connected target within the network was selected to identify phytochemical binding affinity and their interactions with its active site residues. EGFR (PDB: 6LUB) x-ray crystallographic structure was chosen from the RCSB PDB database (https://www.rcsb.org/), visualized for its missing amino acid, and remodelled by homology modeling approach with Uniport ID: P00533 as a query sequence. [124] A total of 100 structures were generated of which the structure with the least DOPE score and the least RMSD value was chosen for further structural refinement using MD simulations.

3.3.4. Least potential energy (PE) conformation by molecular dynamics (MD) simulation

We used Desmond molecular modeling software *ver.* 6.1[125] for MD simulations. All atom-explicit MD simulation for 50 ns was performed with the OPLS force field. The modeled EGFR structure was solvated using the SPC water model in the cubic box having dimensions of 10Å × 10Å × 10Å with periodic boundary conditions. The system was neutralized by adding six positively charged counterions (Na). Further, to restrain the geometry of water molecules, bond angles, and bond lengths of heavy atoms, the SHAKE algorithm was applied and the Particle Mesh Ewald (PME) method was used to treat long-range interactions. The Lennard-Jones interactions cut-off was set to 10Å. The system was then subjected to a production MD run followed by energy minimization using default parameters *via* pressure (1.01325 bar), and temperature (300 K). The trajectory was analyzed to check the structural stability and to obtain the lowest PE confirmation of the EGFR.

3.3.5. Molecular docking.

Three-dimension structures of each bioactive and known EGFR inhibitor Erlotinib were retrieved from the PubChem database in “sdf” file format and converted into “PDB” file format using Biovia Discovery Studio Visualizer 2019. All the small molecules were subjected to energy minimization using the “mmff94” force field in Open babel and the least energy conformation was chosen for docking. The least potential energy conformation of EGFR was extracted from the trajectory and selected for the docking study. Docking was performed using AutoDock vina *via* executed through POAP pipeline.[126] The grid was set around active site residues with box dimensions box center x = 2.8125, y = -9.6422, z = -0.175; and box size 26Å in

all directions with spacing 1Å. The exhaustiveness was set to 100. Docking results were analyzed using a discovery studio visualizer to infer the intermolecular interaction of bioactives with the EGFR target.

3.3.6. Protein-ligand complex stability.

The docked complexes with the least binding energy and maximum interaction with active site residue were subjected to 100ns MD simulation using similar parameters used for EGFR MD. A total of three replicas of the MD simulation were run to get plausible data from the study using the same starting structure and parameters. Trajectories generated were analyzed to investigate stability and intermolecular interactions.

3.3.7. Drug-likeness and side effects prediction

The MolSoft web server (http://www.molsoft.com) was used to predict the drug-likeness of selected bioactives and Erlotinib. Similarly, ADVER-Pred [127] an online server was used to predict the possible side effects of bioactives and Erlotinib.

3.4. Experimental pharmacology

The collected pods are carefully cleaned in running water, cut, dried in the shade, and ground into a coarse powder using a pulverizer. Then, using a Soxhlet apparatus, they are defatted with petroleum ether to remove the fat. Then the filtered and dried cocoa powder was then extracted using a cold maceration technique using an ethanol: water ratio of 80%:20% over the course of one week.[128] By lyophilizing the final dry extract, the percentage yield was derived using the formula.

$$\%\, Yield = practical\ yield / theoretical\ yield \times 100$$

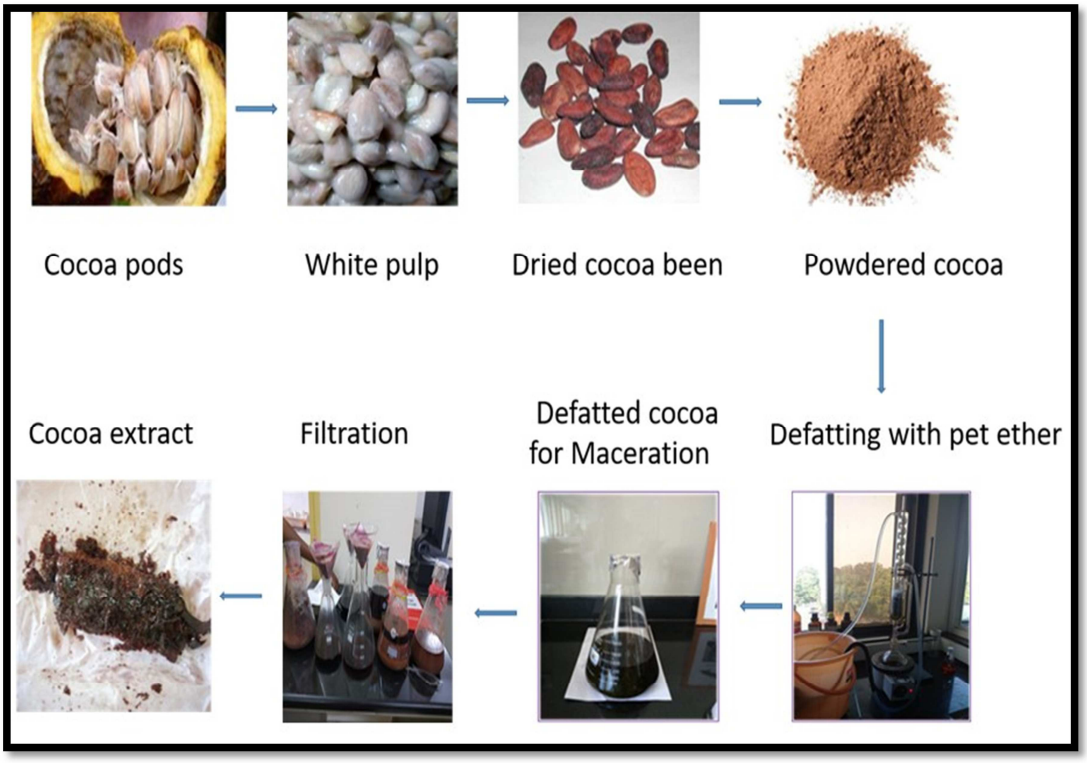


Figure 14: Preparation of extract

4.4.1. HPTLC quantification of cocoa. [129]

I. Layer of chromatograms.

Silica 60 F254 (alugram), 20 × 10 HPTLC plates

II. Sample preparation

One gram of the finely ground, defatted cocoa powder was extracted using ethanol by maceration.

III. Standard solutions

Theobromine, (-)-epicatechin (EC),

IV. Sample application

Using an automatic TLC sampler, band by band. Between 5 and 10 µL for standard solutions and 5 µL for the sample, the solution should be applied.

V. Chromatography

Ethyl formate, formic acid, water, and toluene are mixed in the following proportions: 30:4:3:1.5, calculated for 10 mL to the migration duration of 70 mm (from the bottom edge), for 20 minutes, followed by five minutes of drying.

VI. Derivatization via chromatography

The plates were heated to 100 °C, immersed in Fast Blue Salt B solution (140 mg Fast Blue Salt B mixed in140mL methanol, water 10 mL, and 50 mL dichloromethane), using a Chromatogram Immersion Device (immersion speed 5 cm/s, immersion duration 0 s) and dried for thirty seconds in a cold air stream.

VII. Documentation

TLC Visualizer under white light, as well as under UV 254 nm and after derivatization.

4.4.2. LC-MS of the COE[130]

When running samples through the LC-MS 2010A, the following requirements were checked and confirmed. The stationary phase was column C18, and the mobile phase was methanol and water in a ratio of 90:10. (flow rate 200 L min-1). Record the absorbance at 254 nm after dissolving the sample in the mobile phase and injecting (volume 5 l). The substances in the sample are identified using electrospray ionisation peaks.

4.4.3. Phenol content:

The modified Foin-Chiocalteu method was used to quantify the amount of total phenol. [131]The COE/standard was dissolved into 2 mL of Folin-Ciocalteu reagent (1:10 v/v water dilution) and 2 mL sodium carbonate. To produce colour, tubes were

centrifuged at 1000 x g for 15 minutes and left to stand at 25 ° C for 20 minutes. Using a UV spectrophotometer, the absorbance was measured at 760 nm (Shimadzu, Japan). Total phenolic contents are given as GAE per mg of extract.

4.4.4. Flavonoid content:

Zhishen's method was used to calculate the flavonoid content of COE.[132] Ethanol was used to dissolve 100 µg /mL of COE. First, at zero point 4mL of distilled water is mixed with 1mL of the COE then 0.3mL of NaNO3 (5%) was added. After 5 minutes, 0.3 mL of 10% AlCl3 was added, then at the 6^{th} minute, 2 mL of 1 M sodium hydroxide was added to make up the volume of 2.4mL of water was added. Followed by At 510 nm, the absorption of the pink colour was observed in comparison to the blank value. Standard quercetin solutions (20 to 100 g/mL) to plot the standard curves of total flavonoids. The amount of flavonoids in an extract is expressed as quercetin equivalent (QCE) per milligramme of extract.

4.4.5. DPPH

DPPH radical scavenging activity of the COE was carried out as explained by Williams *et al.,* [133]. DPPH solution was prepared (0.1M, 3.5ml), and it was incubated (25◦C,30min in dark) with multiple concentrations (20 to 100 µg/mL) of the COE in ethanol With control (absence of test agent) after 30 min absorbance of mixtures was recorded at 517 nm. Ascorbic acid was utilized as a standard. The effect of quenching on the %of DPPH was measured by the following equation:

$$DPPH(\%) = \frac{A0 - A1}{A0} \times 100$$

Where A0 and A1 denote the absorbance of the control and test

4.4.6. Nitric Oxide (NO) radical scavenging assay

NO released from sodium nitroprusside was measured as explained by Marcocci *et al.,* [134]. Multiple concentration of COE 1ml was incubated with $Na_2[Fe(CN)_5NO].{}_2H_2O$ (10 mM, 1 mL) for 2.5hr at 25◦C followed by addition of Griess reagent(1.0 mL, composes $C_6H_8N_2O_2S$ (1 %), $C_{12}H_{16}C_{l2}N_2$ (0.1 %), H_3PO_4 (2%)) Then reaction mixture was transferred to a 96-well plate. Absorbance was quantified at 546 nm Gallic acid was a positive control.

$$\text{Nitrogen oxide scavenged (\%)} = \frac{A0 - A1}{A0} \times 100$$

Where A0 and A1 denote control and test

4.4.7. In vitro cytotoxicity assays:

4.4.7.1. Procurement of cell lines and their maintenance

The NCCS, Pune provided the Chinese Hamster Ovary (CHO) and Ehrlich Ascites Carcinoma (EAC) cell lines, which were obtained and cultured in DMEM supplemented with FBS 10%. 20 g/mL penicillin (100 U), and 100 g/mL streptomycin. The cells were subcultured and kept in flasks (T25) at 37°C with 5% CO_2 in a humid incubator until they achieved 70% confluence.

4.4.7.2. Preparation of test samples

The COE was first prepared as a 10 mg/mL stock solution in sterile water with 5% DMSO and filtered through a 0.22 M syringe filter. 100–1000 g/mL of the prepared stock solution was collected and utilised for the test. Each experiment was carried out three times.

4.4.7.3. MTT assay of COE and doxorubicin on EAC and CHO cell lines

The cytotoxic activity of COE and DOX on EAC and CHO cell lines was determined using MTT assay[135] Tetrazolium dye, which is a water-soluble dye with a yellow hue, is reduced to formazan crystals in the MTT assay. During the assay, cell lines were plated onto 96-well flat-bottom plates at a cell density of 20,000 cells/well, and the cells were allowed to grow for 24 h. The stock solutions of COE in 5% DMSO and DOX were prepared at 1 mg/mL in normal saline. Then the cells were treated with COE and DOX. The final volume in each well was made up to 250 µL with DMEM media supplemented with 3% FBS and incubated for 48 h at 37˚C in 5% CO_2. After 48 hr, 20µLof MTT reagent (5 mg/mL stock solution) was added and incubated (4h, 37˚C:5% CO_2). After incubation, wells were washed with PBS thrice to remove the MTT. The MTT reduction product (formazan crystals) was then dissolved in 100 µL of 99.5% DMSO by gentle shaking and the absorbance was noted at 570 nm using an ELISA plate reader. The cytotoxic activity was expressed as a percentage of cell viability in CHO and EAC cell lines compared with the control i.e. extract-treated vs untreated and Doxorubicin treated vs untreated.

4.4.7.4. Chemosensitivity assay[136]

Studies were carried out in Chinese Hamster Ovary (CHO), Ehrlich's Ascites Carcinoma (EAC), and A549 cell lines. All three cell lines were used for chemo-sensitivity assay using various concentrations of COE (2.5 µg/mL to 2560 µg/mL), DOX (0.125 µg/mL to 128 µg/mL) and a combination of both (1.25 to 1280 µg/mL COE + 0.0625 to 64 µg/mL DOX) for 48 hours and cell viability was assayed using MTT (as explained in cell proliferation assay below). The data were normalized and plotted.

4.4.7.5. Cell proliferation assay[136]

All three cell lines were utilized for cell proliferation assay (Kumar P et. al., 2019). Briefly, cells were treated with DOX (1 µg/mL), COE (40 µg/mL) and combination of DOX (0.5 µg/mL) with COE (20 µg/mL) along with control (DMSO) for 48 hours. Later, cells were detached using Trypsin-EDTA solution, collected, counted and plated for all the assays. For the cell proliferation assay, in a 96-well plate (in triplicate) 2000 cells per well were seeded (in triplicate). MTT (Sigma: M5655-1G) was added 24, 48 and 96 hours after seeding (for triplicate samples) for each treatment group and after incubation, for 4 hours at 37 ^{o}C the absorbance was recorded at 570 nm. The data were normalized and plotted.

4.4.7.6. Colony formation assay[136]

Everything for the colony formation assay was carried out similarly to the cell proliferation assay, except for the seeding density, which was 200 cells per well seeded in 24 well plates in duplicate and cultured for 2 weeks. In addition to counting and plotting colonies with at least 50 cells, images were also taken and shown.

4.4.7.7. Wound healing assay[136]

For the wound-healing assay, 0.2 X 10^6 cells per well were seeded in a 24 well plate. Cells were treated by the above-mentioned concentration of samples (test) and the migration was analyzed by measuring the wound size over the various time points. The data were normalized as per the wound size at zero hours and plotted.

4.4.8. In vivo pharmacology

4.4.8.1. Ethical Clearance and Animal Procurement

The IAEC of the ICMR-NITM, Belagavi, India examined and approved the study protocol. (accession number: IAEC/ICMR-NITM BGM/2018/3) for experimentation with animals. Healthy Balb/c female mice (22–25 g) were procured from M/s in vivo Biosciences Bangalore (Karnataka, India) and maintained under controlled conditions of temperature (T:23 ± 2 ◦C H:50 ± 5% and L:12h L/12h D)at the Animal Research Facility, ICMR-NITM, for experimentation.

4.4.8.2. Animal dose selection:

Theobrama cacao acute toxicity studies have been documented in previous publications and the IAEC has recommended using the same data. In these investigations, we used the safer therapeutic dose of 1/10 dose of 2000 mg/kg.[137]

4.4.8.3. Induction of tumor

Mice carrying Ehrlich ascites carcinoma (EAC) tumor cells were obtained from M/s In vivo Biosciences Bangalore (Karnataka, India), and these cells were were kept alive and multiplied by repeated, aseptic intraperitoneal implantation of 1×10^6 cells into other mice (control). The 14-day propagation period produced the cells, which were used in the study.

4.4.8.4. Study 1

4.4.8.5. Survival time in Ehrlich ascites carcinoma tumor model.

A total of 50 female adult Balb/c mice (22–25 g) were randomized using computer-generated random numbers into 5 groups (n = 10), in which animals were injected with EAC except for one group that served as normal and received vehicle (2% gum acacia). Grouping was as (1) Normal: received 2% gum acacia p.o.; (2) EAC: 2% gum acacia p.o.; (3) DOX: received doxorubicin (4.91 mg/kg, i.p., q.wk. calculated based on the therapeutic equivalent dose of doxorubicin)[138] ;(4) COE: received COE 200 mg/kg, p.o.;COE dose was fixed based on acute toxicity studies and pilot safety studies; (5) COE + DOX: received (COE 200 mg/kg, p.o. o.d. + doxorubicin 4.91 mg/kg, i.p., q.wk.) for 28 days and observed daily for survival analysis. The mean survival time[139] was calculated as

$$MST = \frac{\Sigma Survival\ time\ (days) of each\ mouse \in a\ group}{Total\ number\ of\ mice}$$

$$Increased\ life\ span(\%) = \frac{MST\ of\ treated\ mice}{MST\ of\ the\ cancer\ control\ group} X100$$

4.4.8.6. **Evaluation of anticancer, cardioprotective, hepatoprotective, and nephroprotective activity of COE alone and in combination with DOX in the EAC tumor model.**

Fixed-term study

Based on the outcome of the above study, we designed the experiment for 21 days of treatment after tumor inoculation/induction. To study the prophylactic activity of COE on cancer progression and doxorubicin-induced toxicities, we included a COE-pretreated group (Pt). In this group, animals received COE for 21 days before

tumor cell inoculation, and the rest of the treatment was the same as in group 5 (COE + DOX).

4.4.8.7. Animal grouping and experimentation

Sixty female adult Balb/c mice (22–25 g) were randomized using computer-generated random numbers into 6 groups (n = 10), in which animals were inoculated (i.p.) with EAC to induce tumors, except the normal group. Grouping was as (1) Normal: received 2% gum acacia p.o.; (2) EAC: received EAC + 2% gum acacia p.o.; (3) DOX: received doxorubicin (4.91 mg/kg, i.p., q.wk.); (4) COE: received COE 200 mg/kg, p.o.; (5) COE + DOX: received (COE 200 mg/kg, p.o. + doxorubicin 4.91 mg/kg, i.p. q.wk.); (6) Pt + COE + DOX: normal animals pretreated with COE (200 mg/kg, p.o.) for the previous 21 days, EAC was induced on the 22nd day after receiving (COE 200 mg/kg, p.o. + doxorubicin 4.91 mg/kg, i.p., q.wk.) for another 21 days.

During treatment, the body weight of each animal was measured at an interval of 3 days until the end of the study. On the 22nd day, the electrocardiogram (ECG) of individual animals from each group was recorded under anaesthesia with diazepam (3 mg/kg). [140] After successful completion of the ECG record, blood was drawn into 2 tubes, one in an EDTA tube for CBC and another in a microcentrifuge tube for serum separation and estimation of various parameters. Animals were finally euthanized using ketamine overdose, and tumor volume and weight of the vital organs (heart, liver, and kidney) were recorded. Furthermore, portions of each heart, liver, and kidney tissue were homogenized and used for the antioxidant assay. The remaining tissue was fixed in a 10% formalin solution for histopathological studies.

4.4.8.8. Electrocardiogram

Electrocardiograph (ECG) recordings were considered for the diagnosis of cardiac abnormalities in doxorubicin-treated mice at the end of the study. Mice were anaesthetized with diazepam (3mg/kg)[140] and ECG was carried out. The electrocardiograms were recorded using the MP35, Biopac 4.0 system. Briefly, after anaesthesia, the mouse was fixed on a foam plate, ECG patches were placed on the mouse's leg and arm, connected with electrodes, and the two leads were used to collect signals. The red electrode was connected to the upper left limb, the black electrode was connected to the lower left limb, and the white electrode was connected to the right forelimb. ECG was recorded for at least 60s. The ECG recording speed was 30 mm/s and the voltage was 1 mV/cm. ECG wave analysis was performed to calculate heart rate (beats/minute) and QRS complex (ms) using the acqknowledge software.

4.4.8.9. Evaluation of EAC volume and percentage change in body weight

The change in body weight (BW) was recorded at intervals of three days until the completion of an experiment. After 24 h from the last treatment, the mice were sacrificed, and fluid (ascitic) was aspirated from the mice to estimate the EAC volume.

4.4.8.10. Serum biochemical parameters

Last day of the experiment, blood was taken from the medial canthus of the eye of the mice in all groups. Blood was collected in two tubes, one in the EDTA tube for haematological evaluation and another for separation of the serum for biochemical analysis of heart, liver, and kidney function. Cardiac biomarkers creatinine kinase-

MB (CPK-MB) and lactate dehydrogenase (LDH); serum lipid profiles, such as TG: TC: LDL: HDL; the liver markers AST: ALT: ALP and creatinine and blood urea nitrogen for kidney markers were quantified using commercially available kits (Biosystems, S.A., Barcelona, Spain) in a semi-auto analyser (A15 Biosystems S.A., Barcelona, Spain).

4.4.8.11. Haematological evaluations

Complete blood counts White blood cells (WBC), platelets, per cent haemoglobin, mean corpuscular haemoglobin (Corpuscular volume (MCV), mean cell haemoglobin concentration, and packed cell volume are all components of red blood cells (RBC). were measured using an automatic haematology analyser (Erba H560).

4.4.8.12. Quantification of Enzymatic and Non-Enzymatic Antioxidant Biomarkers in the Tissue Homogenate (Heart, Liver, and Kidney)

Mouse heart, liver, and kidney homogenates were tested to quantify multiple enzymatic and nonenzymatic antioxidant parameters. Organs were dissected out and washed in cooled saline, weighed, and homogenized in cold PBS (0.05 M, pH 7.4). The solution was centrifuged (10,000 rpm: 10min: 4◦C), and post-mitochondrial supernatant (PMS) was used to assess TC and LPO[141]. The supernatant was again centrifuged (15,000 rpm: 4◦C: 1 h). for (SOD)[142], catalase (CAT)[143], glutathione (GSH)[144] and total thiols.[144]

4.4.8.13. Histopathological Investigations of the Heart, Liver, and Kidney

Three samples of heart, liver and kidney tissues from each group of mice were fixed in a 10% formalin solution for histopathological studies. The tissue slices were examined at 40× magnification using eosin haematoxylin dye.

4.4.8.14. Study 2

Solid Tumor Model

4.4.8.15. Tumor cell transplantation and grouping of animals

Ehrlich ascites carcinoma cells carrying donor mice were obtained from in vivo biosciences, Bangalore, India and were maintained and propagated by serial intraperitoneal transplantation (1×10^6 cells) into healthy mice in an aseptic environment and propagated for 14 d [145] Ehrlich ascites carcinoma cells were received from donor mice were suspended in sterile saline. A viable number of cells (~ 2×10^6 cells) were injected subcutaneously into the thigh region of the right hind limb.[139]

In the present study, a total of 80 animals were randomly divided into 5 groups (n=16) of which group one served as normal (n=16) and the rest were induced tumors by injecting EAC cell (2×10^6) (suspended in 0.2 mL saline/mice) subcutaneously. Tumor size of all EAC inoculated mice crossed the 50 mm^3 was conceder as day 0 (11^{th} day after implantation)and treatment was initiated Tumor size was measured using a vernier caliper every week and calculated using the following equation as mentioned by Schirner *et al.,*[146]

$$Tumor\ volume = longest\ diameter\ X\ shortest\ width\ X\ 0.5$$

The study included the animals grouping as Normal: vehicle (2% gum acacia OD), EAC: vehicle (2% gum acacia p.o., OD), DOX: EAC+ doxorubicin 4.91 mg/kg,

i.p., qw, COE200: EAC+ Cocoa 200 mg/kg, p.o., OD, COE200 +DOX: EAC+ doxorubicin 4.91 mg/kg,i.p., qw + cocoa 200 mg/kg, p.o., OD.

The treatment was carried out for 21 days and 6 animals from each group were randomly separated, blood samples were collected separately for haematological and biochemical analysis. Further mice were euthanized with an overdose of ketamine to collect the tumor mass and vital organs (heart, liver, and kidney) for antioxidant and histopathological studies. Whereas the rest of the animals (n=10 per group) were kept for survival analysis for up to 60 days. The mean survival time was calculated as explained by[139] as follow

$$MST = \frac{\Sigma Survival\ time\ (days)\ of\ each\ mouse \in a group}{Total\ number\ of\ mice}$$

$$Increased\ life\ span\ (\%) = \frac{MST\ of\ treated\ mice}{MST\ of\ the\ cancer\ control\ group} X\ 100$$

4.4.8.16. Measurements and methods

Bodyweight and tumor size were measured once a week as mentioned previously. A total of 6 mice from each group were selected randomly and blood, vital organs and tumor mass were collected as mentioned before. Further, we evaluated multiple biochemical parameters i.e. Creatinine kinase-MB, lactate dehydrogenase, AST, ALT, ALP, creatinine, and urea using commercially available kits(Biosystems) using a semi-automated analyzer (A15 Biosystems). Also, we evaluated the anti-oxidant biomarkers i.e. catalase[143], SOD[142], GSH[144] and LPO[141] The Haematological parameters such as Complete blood counts were measured using an automatic haematology analyser (Erba H560).

For histopathological analysis, tissue was blinded, sectioned the samples (heart, liver, kidney, and tumor) of 40 µM, fixed in formalin (10%), and stained using hematoxylin and eosin and changes were observed under the microscope.

4.4.9. Statistics

For, in silico pharmacology, the protein-protein interaction regulated KEGG pathways were interpreted based on the gene count and the false discovery rate. The bioactives proteins-pathways interaction was evaluated based on edge count. Lead hits of particular activity were interpreted using binding energy (kcal/mol) and H-bond interaction(s). All the experimental data were presented in mean±SD/SEM wherever applicable. One-way or two-way analysis of variance (ANOVA) followed by posthoc Tukey's test was used to evaluate the quantitative data. Both quantitative and categorical data were evaluated using GraphPad Prism.

5. RESULTS

The results of the study entitled “Pharmacological Evaluation of Cocoa on Efficacy and Toxicity of Doxorubicin in Murine Ascites and Solid Tumor’’ were discussed under the following headings.

5.1. Physico-chemical analysis of cocoa

Table 3: The physic-chemical constants of cocoa were found to be as mentioned in

Parameters	Mean ± SD
Total Ash value	3.433±0.1258 (%)
Water-soluble ash	0.95±0.070 (%)
Acid insoluble as	0.005±0.005 (%)
Alcohol soluble extractive	28.9g in 100g crude powder
Water-soluble extractive	10g in 100g crude powder
Moisture content	7.7±0.43(%)
Crude Fibre Content	10.58±0.52 w/w
pH of cocoa	6.57

5.1.1. Fluorescence analysis

After treatment with different chemical and organic reagents, the fluorescence of T.cacao powder was examined under extended ultraviolet light. The behaviour of fluorescence was observed.

Table 4: Fluorescence analysis

Sample	Short wavelength (254nm)	Long-wavelength (366nm)	Visible light
Powder	Brown	Black	Brown
Powder + conc HCL	Brown	Black	Brown
Powder + conc HNO3	Black	Black	Black
Powder + H2SO4	Black with white Florence	Black with white Florence	Yellowish Brown
Powder +1N NaOH	Black with green Florence	Black	Green
Powder + 1% Picric acid	Black with green Florence	Greenish brown	Yellowish-brow
Powder + Fecl3	Black	Black	Black

5.2. The yield of Extraction and Preliminary Phytochemical Analysis of the Extracts

The yield of the COE was 5.8 per cent w/w of the dried cocoa bean. Phytochemical assessment is carried out to qualitatively detect various chemical components that will assist in monitoring the presence of active ingredients that will cause a strong pharmacological response. The active components of carbohydrates, alkaloids, flavonoids, proteins, amino acids, and phenols were found in T. cacao extracts after analysis.

5.3. HPTLC

The developed method demonstrated good separation and rapid quantification of (Epicatechin) and alkaloid (Thiobromin) Content in the fresh cocoa beans is Thiobromin 60.1±2.9 mg/gm and Epicatechin 50.8 ± 1.03 mg/gm.

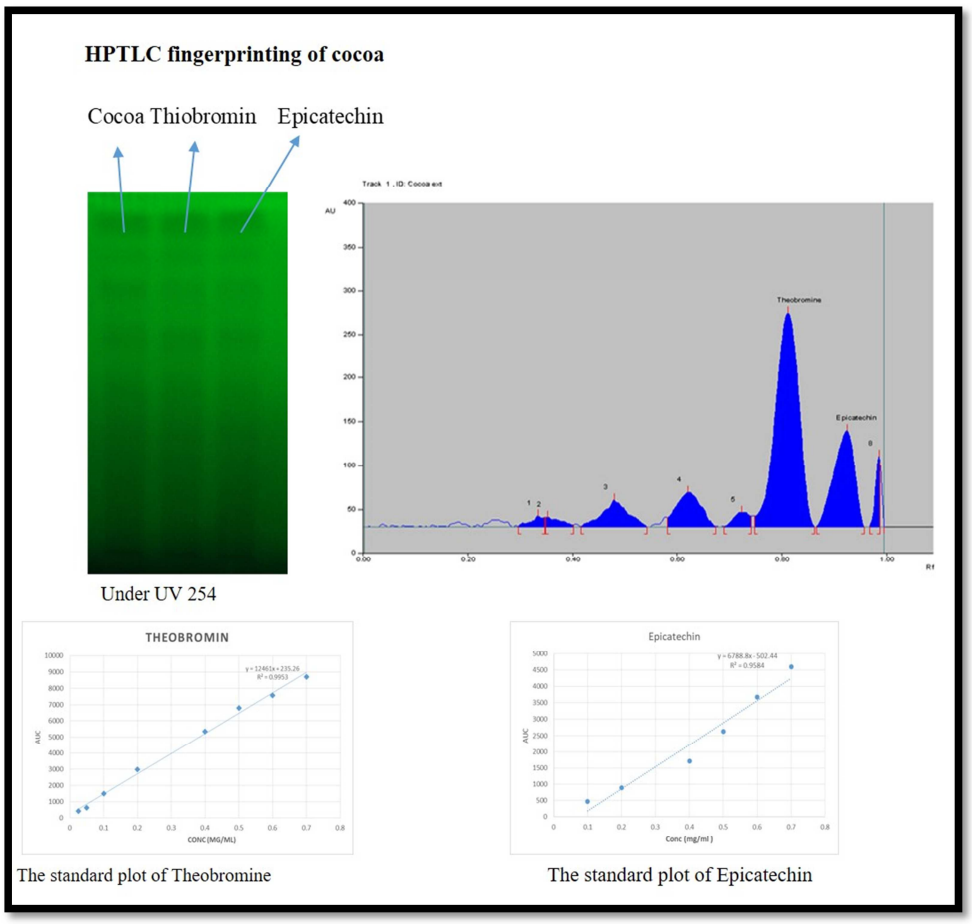


Figure 15: HPTLC fingerprint of cocoa:

5.4. LCMS

The existence of active principles in the network pharmacology was also detected by the LCMS profile of cocoa bean extract (COE). Apigenin is one of the lead hit bioactives found in the LCMS study with a good drug-likeness score. (MF: $C_{22}H_{18}O_{10}$, MW: 442.4), apigetrin (MF: $C_{21}H_{20}O_{10}$, MW: 432.4), Thermopsoside

(MF: $C_{22}H_{22}O_{11}$, MW: 462.4), cinaroside (MF: $C_{21}H_{20}O_{11}$, MW: 448.4), esculetin (MF: $C_9H_6O_4$, MW: 178.14), ferulic acid (MF: $C_{10}H_{10}O_4$, MW: 164.16), hirsutrin (MF: $C_{21}H_{20}O_{12}$, MW: 464.4), kaempferol (MF: $C_{15}H_{10}O_6$, MW: 286.24), luteolin (MF: $C_{15}H_{10}O_5$, MW: 270.24), quercetin (MF: $C_{15}H_{10}O_7$, MW: 302.23), and Quercetin 3-galactoside (MF: $C_{21}H_{19}O_{12}$, MW: 463.09); Figure:16

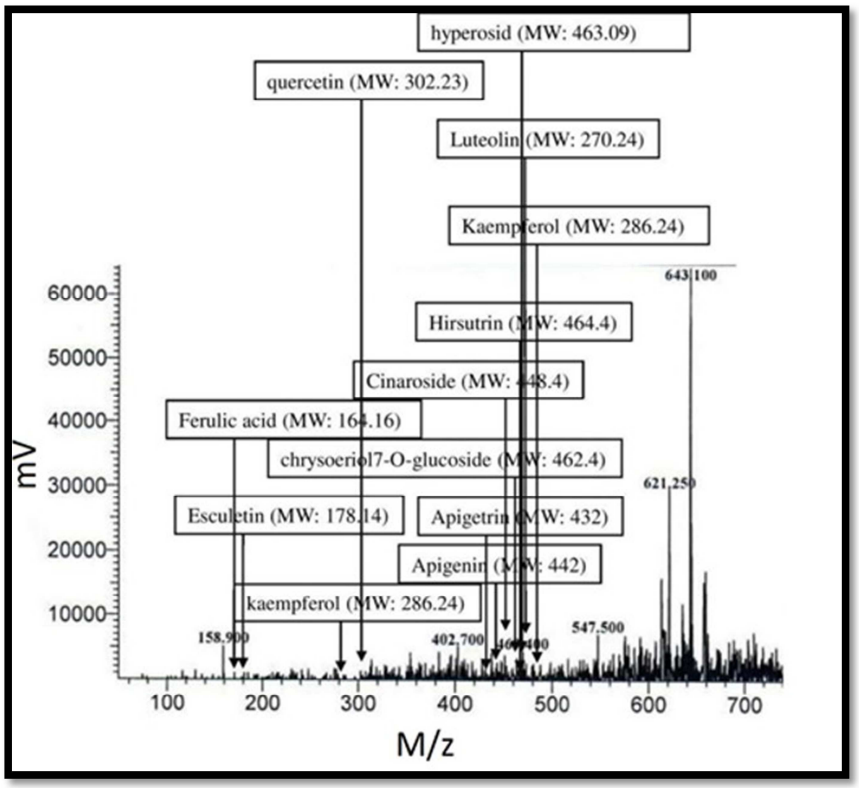


Figure 16: LC-MS analysis of COE

5.5. In silico

5.5.1. Bioactives mining and targets prediction

54 phytocompounds reported in cocoa were retrieved from existing phytochemical databases and literature reviews (Table 5). The majority of the phytocompounds found have been classified as flavonoids and phenols. 34 of the 54 phytocompounds were designed to modulate 220 BindingDB protein targets.

Table 5: List of compounds selected from cocoa = 54

No	Phytoconstituents	MF	MW(g/mol)	HBA	HBD	Log p
1.	Theobromine	$C_7H_8N_4O_2$	180.16	3	1	-0.51
2.	Caffeine	$C_8H_{10}N_4O_2$	194.19	3	0	-0.31
3.	Theograndin II	$C_{21}H_{18}O_{16}S$	558.4	16	8	-2.27
4.	Ethylpyrazine	$C_6H_8N_2$	108.14	2	0	0.64
5.	7-Methylxanthine	$C_6H_6N_4O_2$	166.14	3	2	-0.72
6.	Cinaroside	$C_{21}H_{20}O_{11}$	448.4	11	7	-0.07
7.	Chrysoeriol 7-O- Glucoside	$C_{22}H_{22}O_{11}$	462.4	11	6	0.28
8.	Hirsutrin	$C_{21}H_{20}O_{12}$	464.4	12	8	-0.64
9.	Hyperin	$C_{21}H_{20}O_{12}$	464.4	12	8	-0.64
10.	Guaijaverin	$C_{20}H_{18}O_{11}$	434.3	11	7	0.01
11.	Proanthocyanidin A1	$C_{30}H_{24}O_{12}$	576.5	12	9	4.49
12.	Bis-8,8'-Catechinylmethane	$C_{31}H_{28}O_{12}$	592.5	12	10	3.33
13.	Cosmosiin	$C_{21}H_{20}O_{10}$	432.4	10	6	0.31
14.	Vitexin	$C_{21}H_{20}O_{10}$	432.4	10	7	0.17
15.	Isovitexin	$C_{21}H_{20}O_{10}$	432.4	10	5	0.17
16.	D-Catechol	$C_{15}H_{14}O_6$	290.27	6	5	1.88
17.	Protocatechuic Acid	$C_{25}H_{48}O_4Si_3$	496.9	4	0	5.83
18.	Theophylline	$C_7H_8N_4O_2$	180.16	3	1	-0.24
19.	Pheynlaianine	$C_9H_{11}NO_2$	165.19	3	3	-0.31
20.	Proanthocyanidin B1	$C_{30}H_{26}O_{12}$	578.5	12	10	3.29
21.	Proanthocyanidin B2	$C_{30}H_{26}O_{12}$	578.5	12	10	3.29
22.	Leucine	$C_6H_{13}NO_2$	131.17	3	3	-0.47
23.	(-) Epicatechin	$C_3H_7NO_2$	89.09	10	7	2.96
24.	Apigenin	$C_{22}H_{18}O_{10}$	442.4	5	3	3.06
25.	Luteolin	$C_{15}H_{10}O_5$	270.24	6	4	2.68
26.	Kaempferol	$C_{15}H_{10}O_6$	286.24	6	4	2.49
27.	Chlorogenic Acid	$C_{15}H_{10}O_6$	286.24	9	6	-0.3
28.	Coumaric Acid	$C_9H_8O_3$	326.3	8	5	-0.68

29.	Ferulic Acid	$C_{10}H_{10}O_4$	164.16	3	2	2.07
30.	Quercetin	$C_{15}H_{10}O_7$	302.23	7	5	2.11
31.	(-)-Salsolinol	$C_{10}H_{13}NO_2$	179.22	3	3	0.88
32.	Ascorbic-Acid	$C_6H_8O_6$	176.12	6	4	-2.4
33.	Niacin	$C_6H_5NO_2$	123.11	3	1	0.51
34.	Oleic-Acid	$C_{18}H_{34}O_2$	282.5	2	1	7.15
35.	Valine	$C_5H_{11}NO_2$	117.15	3	3	-1.02
36.	Theophylline	$C_7H_8N_4O_2$	180.16	3	1	-0.24
37.	Dopamine	$C_8H_{11}NO_2$	153.18	3	4	0.36
38.	Esculetin	$C_9H_6O_4$	178.14	4	2	1.08
39.	Amyl-Butyrate	$C_9H_{18}O_2$	158.24	2	0	3.14
40.	Chlorogenic-Acid	$C_{16}H_{18}O_9$	354.31	9	6	-0.3
41.	Epigallocatechin	$C_{15}H_{14}O_7$	306.27	7	6	1.5
42.	Esculetin	$C_9H_6O_4$	178.14	4	2	1.08
43.	Lecithin	$C_{20}H_{40}NO_8$ P	453.5	8	0	2.91
44.	P-Hydroxy-Benzoic-Acid	$C_7H_6O_3$	138.12	3	2	1.3
45.	Pantothenic-Acid	$C_9H_{17}NO_5$	219.23	5	4	-1.18
46.	Pectin	$C_6H_{10}O_7$	194.14	7	5	-3.01
47.	Protocatechuic-Acid	$C_7H_6O_4$	154.12	4	3	0.93
48.	Riboflavin	$C_{17}H_{20}N_4O_6$	376.4	8	3	-1.75
49.	Serine	$C_3H_7NO_3$	105.09	4	4	-2.92
50.	Stigmasterol	$C_{29}H_{48}O$	412.7	1	1	8.82
51.	Thiamin	$C_{12}H_{17}N_4OS+$	265.36	4	3	0.51
52.	Trigonelline	$C_7H_7NO_2$	137.14	2	0	0.65
53.	Tyramine	$C_8H_{11}NO$	137.18	2	3	0.74
54.	Vanillic-Acid	$C_8H_8O_4$	168.15	4	2	1.27

5.5.2. Network construction

Gene set enrichment analysis determined a sum of 220 targets interact with one another to regulate 104 pathways with reference to the KEGG database. Bioactive-regulated targets of probable protein-protein interaction were represented in Figure 17 in which, 21 pathways were identified to associate with cancer and oxidative stress *via* modulating 50 protein targets. The lowest false discovery rate (FDR) of 1.27E-06 was found in the Arachidonic acid metabolism pathway (hsa00590) by triggering 7 genes (AKR1C3, ALOX12, ALOX5, CYP2C9, PLA2G10, PLA2G2A, and PLA2G5). Similarly, pathways in cancer (hsa05200) scored the second-lowest FDR of 1.34E-06 by modulating 15 genes (AKT1, ALK, AR, EDNRA, EGFR, ESR1, FGFR1, HGF, HSP90AA1, IL2RA, KIT, PGF, STAT1, TERT, and VEGFA). Following the cancer pathways, cAMP, Rap1, Ras, Phospholipase D, cGMP-PKG, MAPK, PI3K-Akt, and VEGF signalling pathways scored the lowest FDR and had potential involvement in oxidative stress and cancer Table 6.

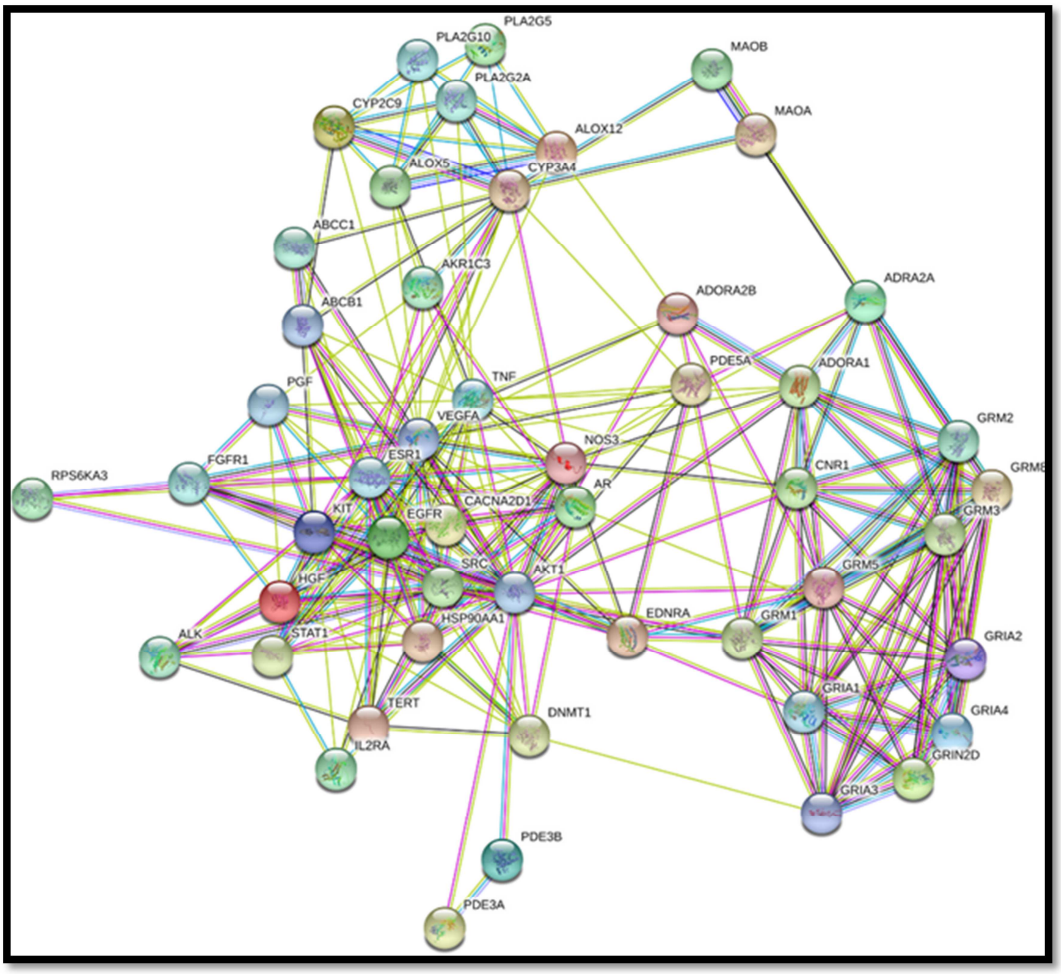


Figure: 17. **Protein-protein interaction of the regulated targets by the bioactives.**

Coloured nodes: Query proteins and first shell of interactors, white nodes: Second shell of interactors, Node content; empty nodes: Proteins of unknown 3D structure, filled nodes: Some 3D structure is known or predicted.

Table.6 Enrichment analysis of pathways involved in oxidative stress and cancer

Pathway ID	Pathway name	Gene count	False discovery rate	Regulated genes
hsa00590	Arachidonic acid metabolism	7	1.27E-06	AKR1C3, ALOX12, ALOX5, CYP2C9, PLA2G10, PLA2G2A, PLA2G5
hsa05200	Pathways in cancer	15	1.34E-06	AKT1, ALK, AR, EDNRA, EGFR, ESR1, FGFR1, HGF, HSP90AA1, IL2RA, KIT, PGF, STAT1, TERT, VEGFA
hsa04024	cAMP signaling pathway	10	1.67E-06	ADORA1, AKT1, EDNRA, GRIA1, GRIA2, GRIA3, GRIA4, GRIN2D, PDE3A, PDE3B
hsa04015	Rap1 signaling pathway	10	2.18E-06	ADORA2B, AKT1, CNR1, EGFR, FGFR1, HGF, KIT, PGF, SRC, VEGFA
hsa04014	Ras signaling pathway	10	5.60E-06	AKT1, EGFR, FGFR1, HGF, KIT, PGF, PLA2G10, PLA2G2A, PLA2G5, VEGFA
hsa04072	Phospholipase D signaling pathway	8	1.36E-05	AKT1, EGFR, GRM1, GRM2, GRM3, GRM5, GRM8, KIT
hsa04022	cGMP-PKG signaling pathway	8	2.38E-05	ADORA1, ADRA2A, AKT1, EDNRA, NOS3, PDE3A, PDE3B, PDE5A
hsa04010	MAPK signaling pathway	10	3.16E-05	AKT1, CACNA2D1, EGFR, FGFR1, HGF, KIT, PGF, RPS6KA3, TNF, VEGFA
hsa05205	Proteoglycans in cancer	8	7.57E-05	AKT1, EGFR, ESR1, FGFR1, HGF, SRC, TNF, VEGFA
hsa04151	PI3K-Akt signaling pathway	10	0.00011	AKT1, EGFR, FGFR1, HGF, HSP90AA1, IL2RA, KIT, NOS3, PGF, VEGFA
hsa01521	EGFR tyrosine kinase inhibitor resistance	5	0.00042	AKT1, EGFR, HGF, SRC, VEGFA
hsa05215	Prostate cancer	5	0.00095	AKT1, AR, EGFR, FGFR1, HSP90AA1
hsa04370	VEGF signaling pathway	4	0.0015	AKT1, NOS3, SRC, VEGFA
hsa00982	Drug metabolism—cytochrome P450	4	0.002	CYP2C9, CYP3A4, MAOA, MAOB
hsa05212	Pancreatic cancer	4	0.0026	AKT1, EGFR, STAT1, VEGFA
hsa05224	Breast cancer	5	0.0039	AKT1, EGFR, ESR1, FGFR1, KIT
hsa05226	Gastric cancer	5	0.0039	ABCB1, AKT1, EGFR, HGF, TERT
hsa05206	MicroRNAs in cancer	5	0.004	ABCB1, ABCC1, DNMT1, EGFR, VEGFA
hsa05219	Bladder cancer	3	0.0048	EGFR, SRC, VEGFA
hsa04923	Regulation of lipolysis in adipocytes	3	0.0084	ADORA1, AKT1, PDE3B
hsa04012	ErbBsignaling pathway	3	0.0228	AKT1, EGFR, SRC
hsa04630	Jak-STAT signaling pathway	4	0.0228	AKT1, EGFR, IL2RA, STAT1

The network between compound-protein (Figure 18), protein-pathway (Figure 19), and compounds-proteins-pathways (Figure 20) were constructed by treating edge count topological parameters using Cytoscape ver 3.6.1. Network analysis identified, among all the queried protein targets involved in cancer and oxidative stress, the EGFR was identified as an enriched hub protein within the network that scored the highest edge count (Figure 20). EGFR was found to involve in 15 pathways out of 21 and targeted by 11 phytocompounds of cocoa based on network analysis, the EGFR was elected to infer the intermolecular interactions with phytocompounds of Cocoa by molecular docking and dynamics analysis.

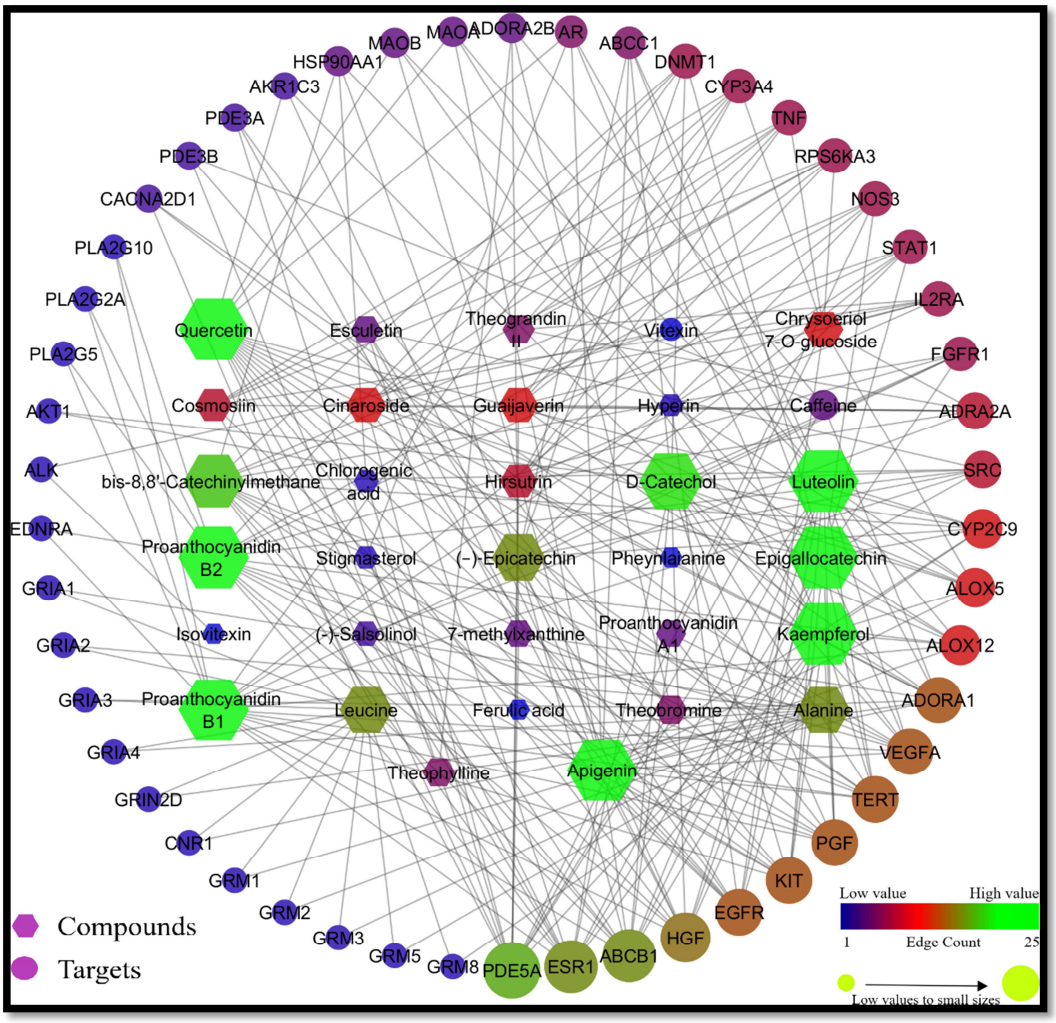


Figure 18: Interaction of phytocompounds with respective predicted targets

A hexagon represents the compounds and a circle represents the targets

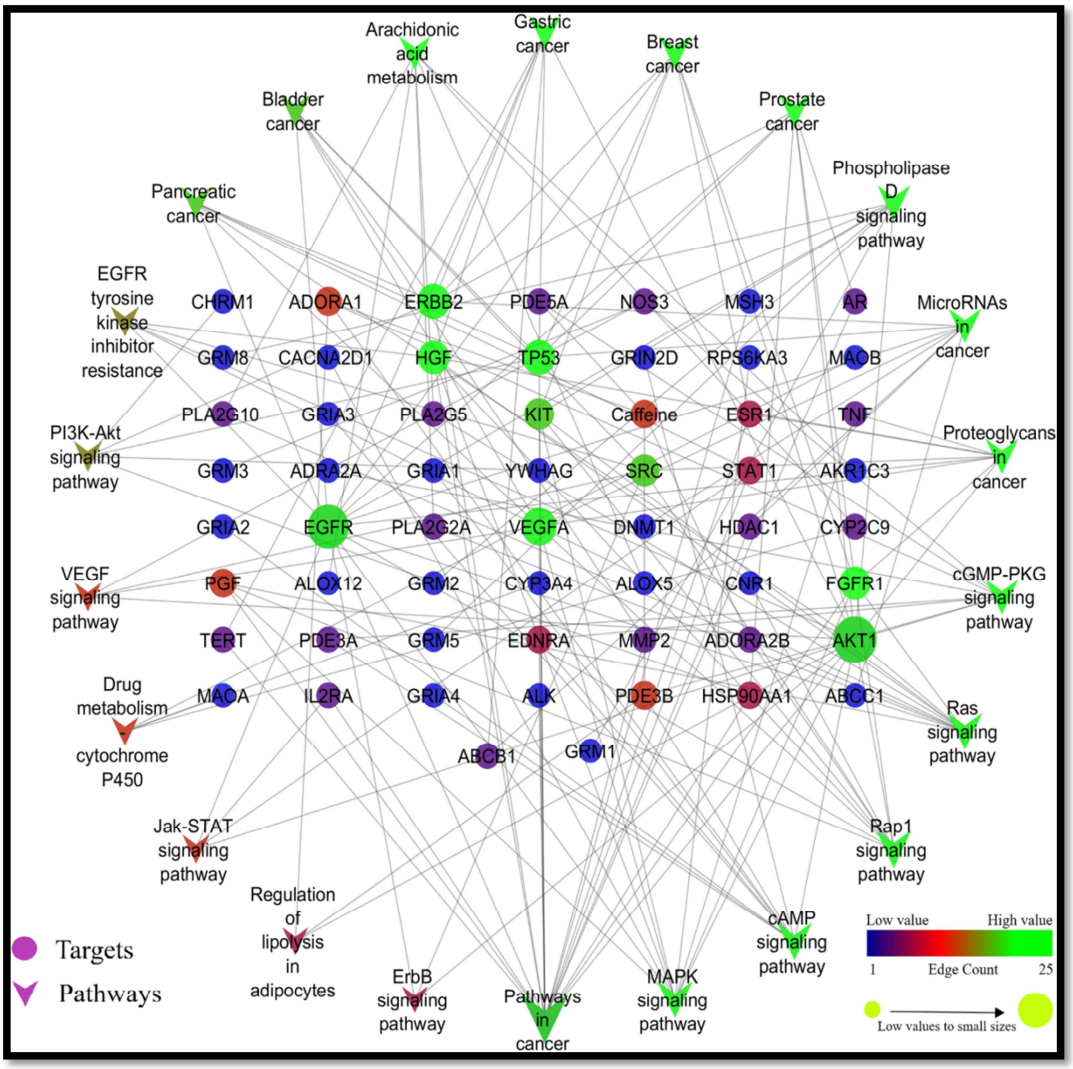


Figure 19: Pathways are regulated and their representative proteins concerning the KEGG database

Arrow represents the modulated pathways and the circle represents the targets

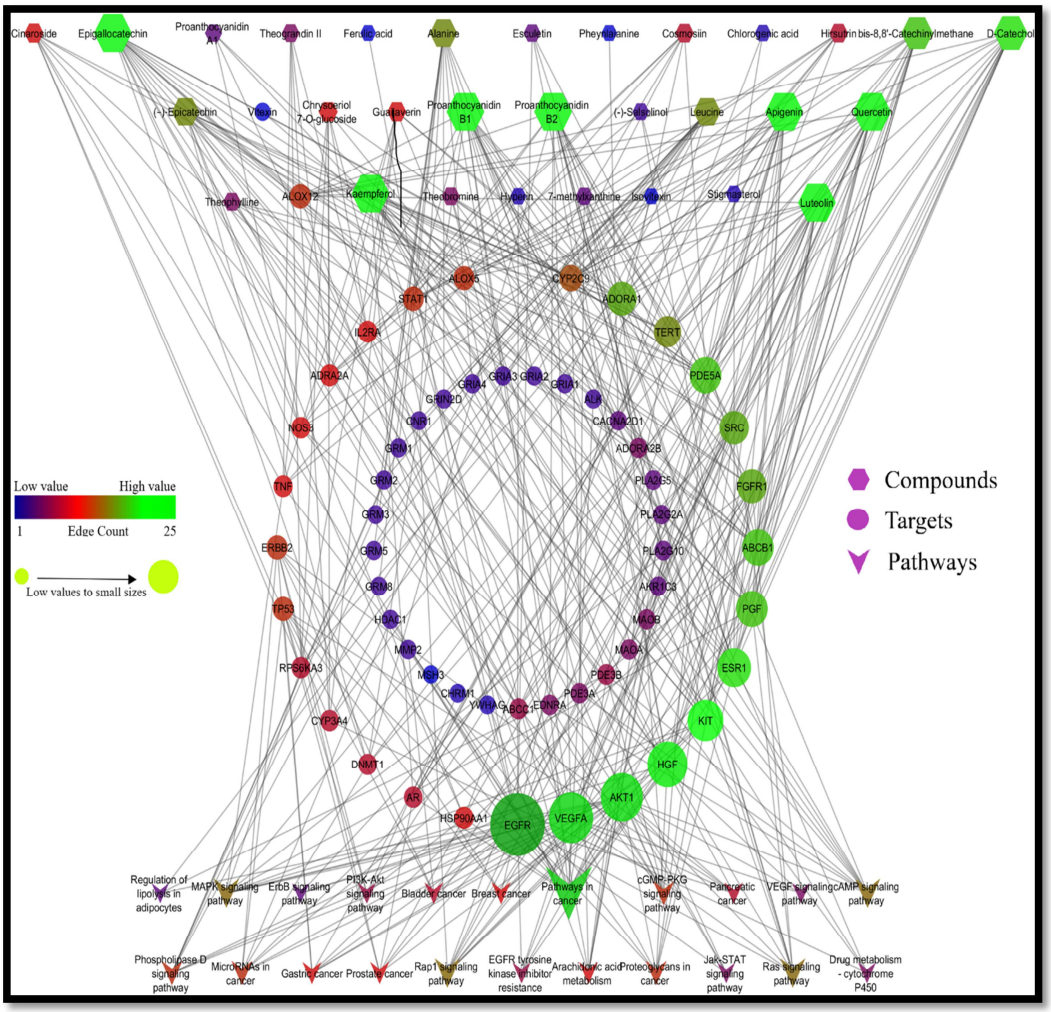


Figure 20: Interaction of the bioactives with their targets and modulated pathway

The hexagon represents the compounds a circle represents the targets, and an arrow represents the pathways

5.5.3. EGFR structure refinement, lowest PE conformation from MD, and its active site residues

A total of 100 models were generated, of which we chose model 63 because it showed the lowest DOPE yield (- 37918.11). The stereochemical properties of the modeled EGFR were analyzed using the Ramachandran plot (Figure 21A). RMSD of 0.359 with a pattern indicating the reliability of the selected model (Figure 21B).

Parameters describing structural stability, such as RMSD and RMSF, showed stable dynamics over the 50 ns simulation (Figures 24A and 24B, respectively). Further, the structure with the lowest potential energy at 33.7 ns was extracted from the MD simulation trajectory and used for docking studies.

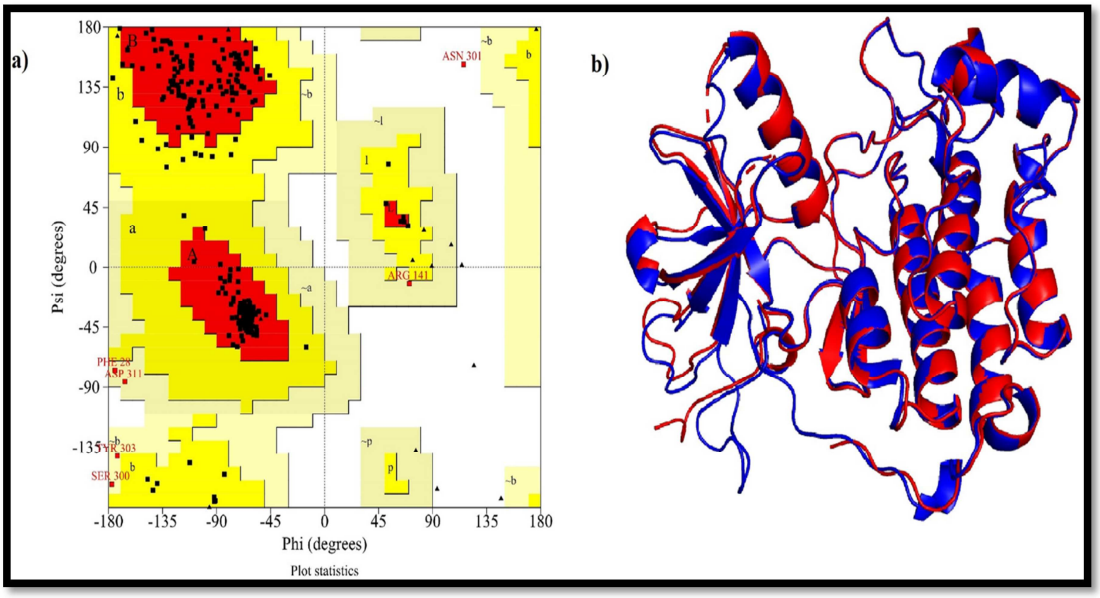


Figure 21: (a) Ramachandran plot of EGFR protein. The red region represents the most favoured region, Dark yellow represents the favoured region, fade yellow represents the allowed region and the white region represents the non-allowed region. **(b) 3D structure of EGFR generated using homology modeling.** Blue represents modeled protein, red represents the template (PDB: 6LUB).

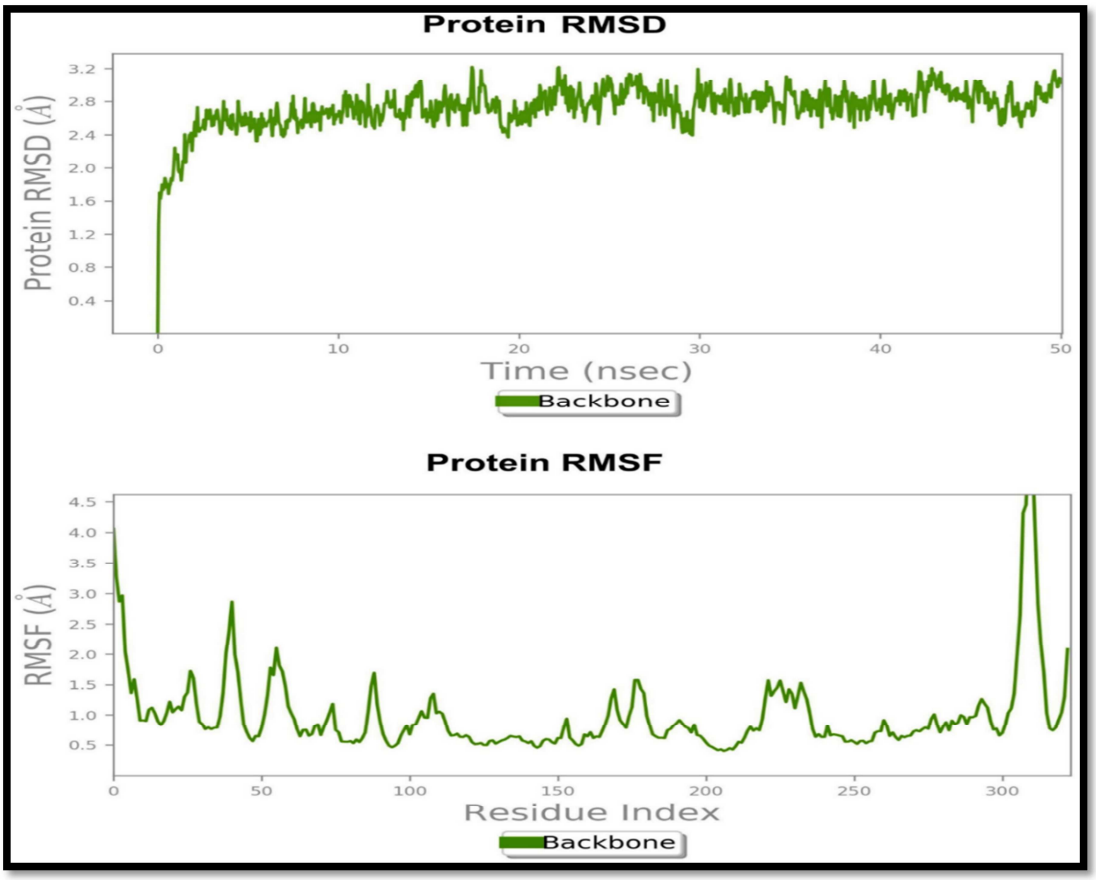


Figure 22: Structural stability of the generated homology model of EGFR protein observed during 50ns MD simulations.

(a) Root mean square fluctuation revealed EGFR reached an equilibration state after ~22 ns and showed stable dynamics thereafter. (b) binding pocket residues Leu144, Ala48, Ser102, Val31, Met98, Cya33, Pro99, Tyr106 etc. showed less residual fluctuations however other flexible loop regions including N and C-terminal residues show maximum residual fluctuations.

5.5.4. Molecular docking

Active site residues of the EGFR, namely Leu23, Lys33, Ala48, Lys50, Cys80, Met95, Gln96, Leu97, Met98, Pro99, Arg146, Asn147, Leu149, Thr159, and Asp160 were extracted from the crystal structure of ‘‘6LUB.pdb’’. Docking studies revealed that all 11 pytocompounds were successfully attached to the EGFR binding site. Among them, hirsutrin (BE = -7.2 kcal/mol), also known as isoquercitrin, had the most stable contacts (11), 8 of which were with the specified binding pocket residue (Figure 23). We found H-binding interactions with Ser102, Phe100, and Lys33, and other non-bonded interactions with Lys33, Leu97, Ala48, Leu149, Leu23, and Phe100. However, Apigetrin, showed the lowest binding affinity with EGFR (-9.0kcal/mol), by forming a maximum of 8 interactions established, of which only 4 interactions are with the active site residues Arg146, Asn147 (2), Lys50. Erltonib shows BE of -7.0 forming two H-bonds with active site residues Asn147 and Lys50. It also formed six nonhydrogen bonds with residues Ala144, Trp185, Gly162, Glu211, and Phe28 (2). Therefore we select Hirsutrin-EGFR for further MD simulation study. Table 7 lists the binding energy of all the docked bioactives and their intermolecular interactions.

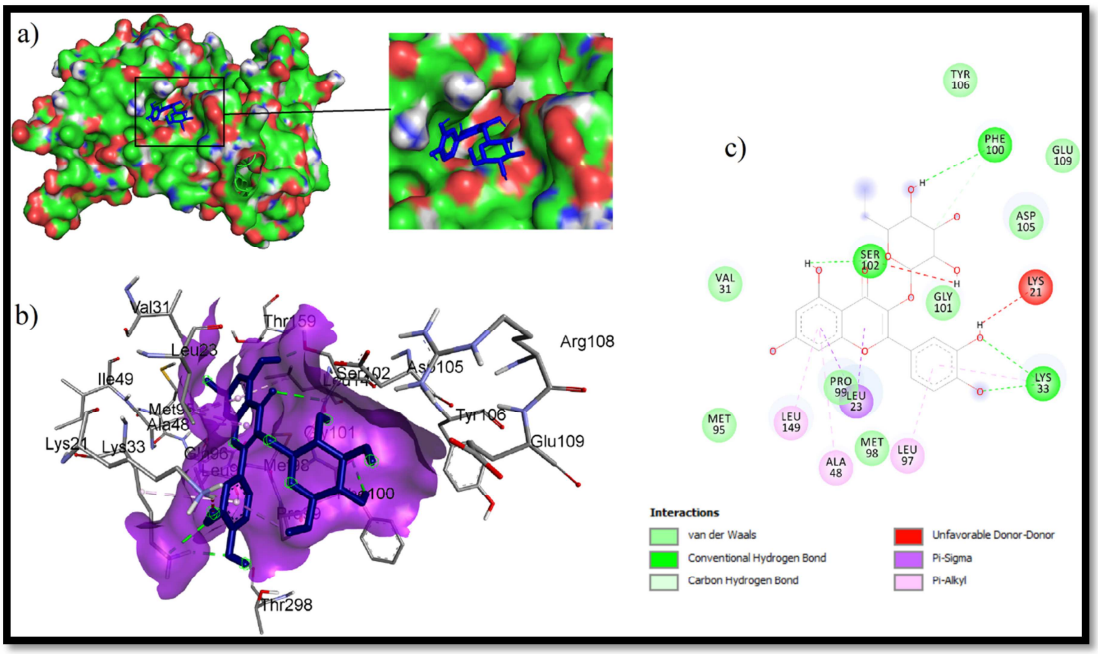


Figure 23: (a) Surface view of EGFR showing ligand binding sites where hirsutrin is deeply buried (refer to inset) into the EGFR binding cavity forming compact globular shape and (b) Intermolecular interactions observed in complex EGFR-Hirstutrin.

Table.7 The binding affinity of compounds with EGFR

Compound name	Compound CID	BE (kcal/ mol)	Conventional HBI	Non-conventional interactions	No. of interactions with active site residues
Apigetrin	5280704	-9.0	Asn147 (2), Arg146	Glu67, Leu52 (2), Ile64, Lys50	4
Cinaroside	5280637	-8.6	Glu211, Arg146 (2), Lys50	Trp185 (2), Ala144 (2), Arg146 (2)	5
Chrysoeriol7-O-glucoside	11294177	-8.3	Arg146, Glu67 (2)	Trp185, Ala144 (2), Arg146 (3), Phe28	4
Kaempferol	5280863	-8.0	Phe28, Arg146	Leu52 (2), Ile64, Lys50, Glu67 (2), Arg163, Gly162, Ile64	2
Quercetin	5280343	-7.8	Nil	Val31, Leu149, Leu23	2
Apigenin	5280443	-7.7	Arg146	Glu67 (2), Leu52 (2), Lys50, Ile64	2
Luteolin	5280445	-7.7	Asp160, Met98	Leu149, Ala48, Val31, Leu23 (2)	6
Hirsutrin	74982342	-7.2	Ser102, Phe100, Lys33 (2)	Lys33, Leu97, Ala48, Leu149, Leu23 (2), Phe100	8
Hyperosid	90657624	-7.2	Ser102, Asp105, Lys33	Leu149, Leu23 (2), Ala48, Phe100	5
Ferulic acid	445858	-6.4	Arg146.O- (2), Met98. . .OH, Met98. . . = O	Leu149, Leu23, Val31, Asp160	7
Esculetin	5281416	-6.2	Leu23, Met98	Gly101, Ala48, Leu149, Leu23 (2)	6

BE, Binding energy; HBI, Hydrogen bond interactions.

5.5.5. MD simulation of the Hirsutrin-EGFR complex

The complex Hirsutrin-EGFR exhibited stable dynamics as revealed by parameters RMSD, RMSF, rGyr, and intermolecular interactions in all three replicas. Backbone EGFR showed a mean RMSD of 1.18Å and 3.22Å between the initial and final frame. The average backbone RMSD for all three replicas was 2.62Å. The complex RMSD was 4.56Å (initial and final frame RMSD was 1.094Å and 4.281Å, respectively). Further, the residue-wise fluctuation was analyzed through RMSF for the protein backbone. The average RMSF of protein residues (322 residues) was 1.20Å. The c- terminal loop residues (300–320) showed maximum fluctuation for ~5.5Å due to the flexibility. Active site residue involved in the interactions *viz.,* Gln96, Met98, and Asp105 showed the least RMSF fluctuation (~1.5Å) in all three replicas throughout the simulation. The ligand means rGyr for three replicates was 4.16Å (initial and final frame rGyr was 4.26Å and 4.144Å, respectively). The overall residual fluctuations of EGFR were very less except for terminal residues and loop regions. The complex Hirsutrin-EGFR formed a complex globular shape as showed by a steady decline in the rGyr values. Figure 24 represents the RMSD of the EGFR backbone (24A) and complex (24B). Figure 25 represents the RMSF of protein (25A) and ligand rGyr fluctuation (25B). Residues Gln96, Met98, and Asp105 show very stable and conserved interactions throughout the simulation period in all three replicates. In replica 1, Met98 showed an interaction fraction of about 53% with hirsutrin whereas, in replicas 2 and3, it showed interaction fractions of 91% and 89% respectively. Similarly, another important binding pocket residue Gln96 showed about 52%, 65%, and 88% interaction fractions in replicas 1, 2, and 3, respectively with hirsutrin.

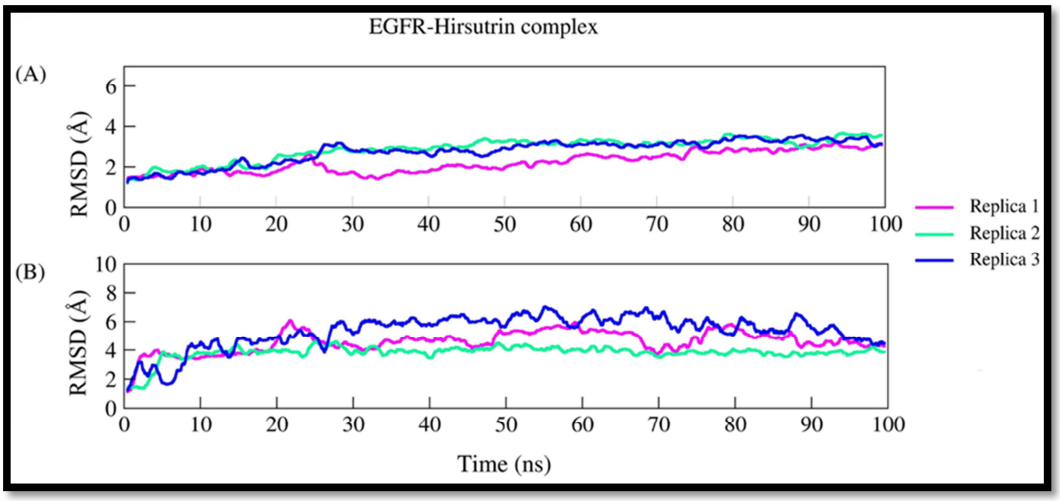


Figure 24: (a) Root mean square deviation of EGFR backbone, and (b) hirsutrin in complex with EGFR

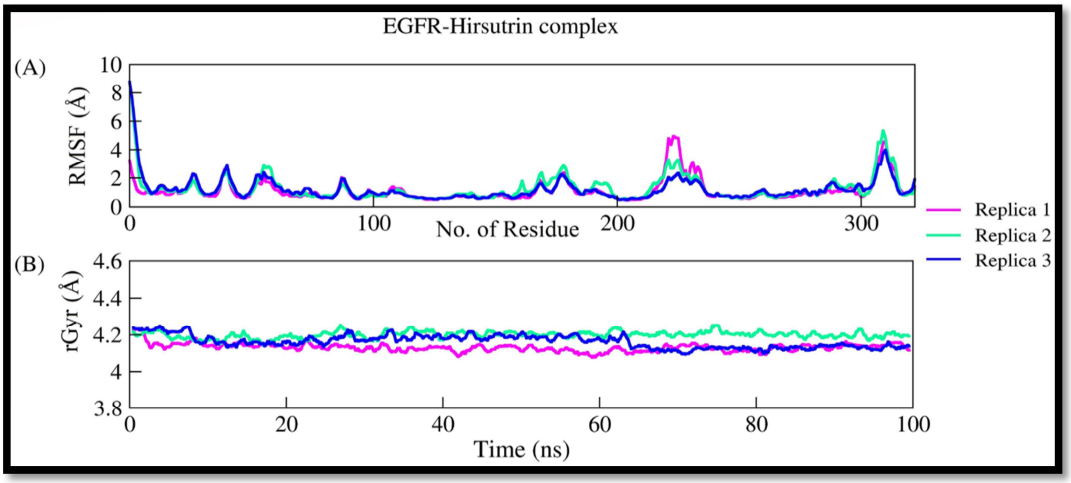


Figure 25: (a) The qualitative parameters explaining structural stabilities like residual fluctuations and (b) radius of gyration in all the replicas

5.5.6. Drug likeness and side effects

All the compounds scored a positive DLS except ferulic acid and esculetin (Table 8). Interestingly, hirsutrin scored the highest DLS of 0.84 which showed stable and maximum interactions in docking and MD simulations. While apigenin and luteolin scored the least DLS of 0.39 and 0.38 respectively. The possible side effect is

predicted using the ADVERpred server. Four compounds namely, apigenin, quercetin, luteolin, and kaempferol scored Pa>0.5 for hepatotoxicity, 6 compounds ferulic acid, cinaroside, hirsutrin, esculetin, chrysoeriol7-o- glucoside, and apigetrin scored Pa<0.5 for hepatotoxicity, nephrotoxicity, and myocardial infarction. A compound hyperosid did not show any probable side effects. Similarly, a control molecule, erlotinib was also predicted to cause hepatotoxicity (Pa = 0.90), myocardial infarction (Pa = 0.665), nephrotoxicity (Pa = 0.594), and cardiac failure (Pa = 0.362).

Table 8: Drug likeness and side effects profile of bioactives targeting EGFR

Cocoa bioactives	MF	MW (g/mol)	NHBA	NHBD	LogP	DLS	Pa	Pi	Predicted side effect(s)
Apigenin	$C_{22}H_{18}O_{10}$	442.4	5	3	3.06	0.39	0.525	0.182	Hepatotoxicity
Apigetrin	$C_{21}H_{20}O_{10}$	432.4	10	6	0.31	0.59	0.38	0.096	Nephrotoxicity
							0.363	0.288	Hepatotoxicity
Chrysoeriol7-O-glucoside	$C_{22}H_{22}O_{11}$	462.4	11	6	0.28	0.56	0.361	0.108	Nephrotoxicity
Cinaroside	$C_{21}H_{20}O_{11}$	448.4	11	7	-0.07	0.6	0.395	0.262	Hepatotoxicity
							0.373	0.1	Nephrotoxicity
Esculetin	$C_9H_6O_4$	178.14	4	2	1.08	-1.22	0.463	0.216	Hepatotoxicity
Ferulic acid	$C_{10}H_{10}O_4$	164.16	3	2	2.07	-0.61	0.44	0.057	Myocardial infarction
Hirsutrin	$C_{21}H_{20}O_{12}$	464.4	12	8	-0.64	0.84	0.387	0.268	Hepatotoxicity
Kaempferol	$C_{15}H_{10}O_6$	286.24	6	4	2.49	0.5	0.525	0.182	Hepatotoxicity
Luteolin	$C_{15}H_{10}O_5$	270.24	6	4	2.68	0.38	0.559	0.165	Hepatotoxicity
Quercetin	$C_{15}H_{10}O_7$	302.23	7	5	2.11	0.52	0.559	0.165	Hepatotoxicity
Hyperosid	$C_{21}H_{19}O_{12}$	463.09	12	7	-0.32	0.64	Not predicted		

MF, Molecular Formula; MW, Molecular Weight, HBA, Hydrogen Bond Acceptor; HBD, Hydrogen Bond Donor; LogP, Partition Co-efficient; DLS, Druglikeness score; Pa, Probable activity; Pi, Probable inactivity.

5.6. Phenol content

Using the calibration curve regression equation, the extract's total phenol content was determined. ($y = 0.0192x + 0.0257$; $R2 = 0.992$), given in mg of gallic acid equivalent (GAE), was 72.5 g gallic acid equivalent /mg extract.

5.7. Total flavonoid content

The total flavonoid content in COE was determined using a calibration curve regression equation ($y = 0.0009x + 0.0237$, $R^2 = 0.9801$), given in mg of quercetin equivalent (QE), and amounted to 165 g QE/mg extract.

5.8. DPPH free *radicals scavenging*

The cocoa extract showed significant activity in scavenging DPPH radicals in a concentration-dependent manner. IC_{50} of the COE was 54.35 ± 0.09 g/mL, while the IC_{50} of standard ascorbic acid molecules was 46.84 ± 1.164 g/mL.

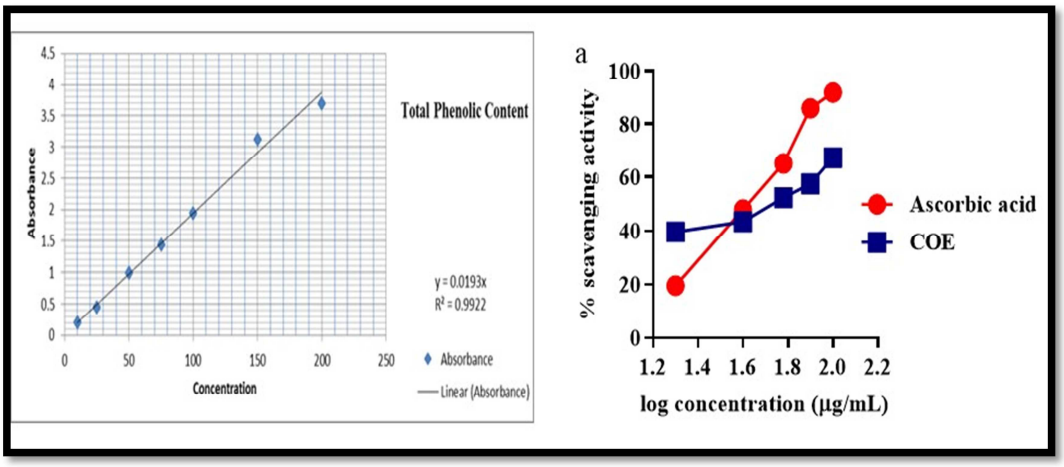


Figure: 26: DPPH radical scavenging activity of COE and ascorbic acid

5.9. Nitric Oxide free radical scavenging assay

The cocoa extract showed significant activity in scavenging Nitric Oxide radicals in a concentration-dependent manner. IC_{50} of the extract was found to be 249.82±36.42µg/mL, whereas, the IC_{50} value of Gallic acid was found to be 125.96±6.41µg/mL.

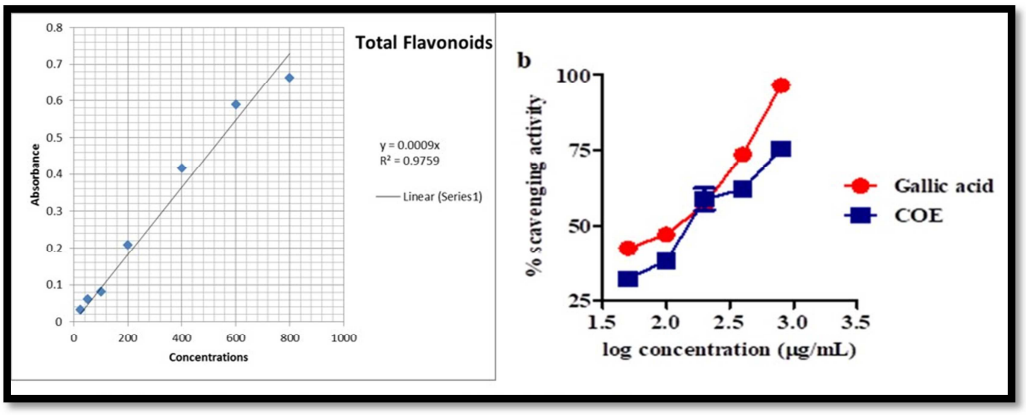


Figure 27: Nitric oxide scavenging activity of COE and Gallic acid

5.10. In vitro cytotoxicity assays

5.10.1. MTT assay

Cytotoxic activity of COE (Figure 28) and DOX (Figure 29) was performed in CHO and EAC. The mean IC_{50} of the cocoa extract against CHO and EAC cells at 24 and 48h was 425.42±5.7µg/mL; 420.15±5.4µg/mL and 210.3±10.93µg/mL; 222.8±0.68µg/mL, respectively. Further, the mean IC_{50} of the DOX against CHO and EAC cells were found to be 4.170±0.20µg/mL; 6.39±0.2 µg/mL and 8.2±0.32µg/mL; 7.4±0.45µg/mL, respectively**.**

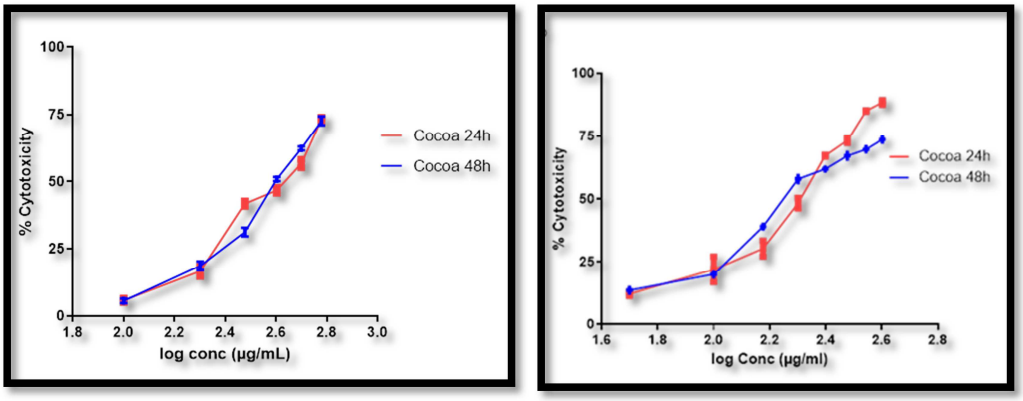


Figure 28: Cytotoxicity of COE in CHO (normal) and EAC (cancer) cell line after 24h and 48 h treatment.

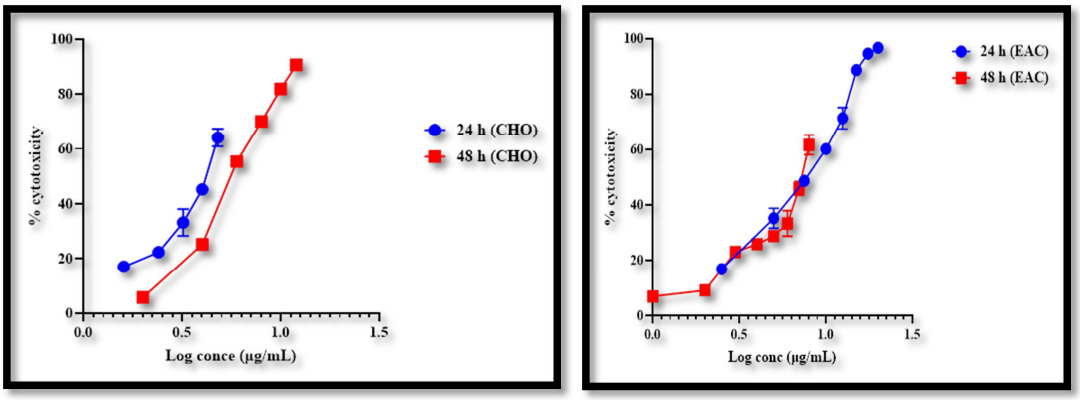


Figure 29: Cytotoxicity of DOX in CHO (normal) and EAC (cancer) cell line after 24h and 48 h treatment.

Table 9. Cytotoxicity of COE and Doxorubicin in CHO and EAC cancer cell line after 24h and 48 h treatment

Cell line	Cocoa extract	Cocoa extract	Doxorubicin	Doxorubicin
	24hr	48hr	24hr	48hr
CHO	425.42±5.7	420.15±5.4	4.170±0.20	6.39±0.2
EAC	210.3±10.93	222.8±0.68	8.2±0.32	7.4±0.45

5.10.2. Cell proliferation assay

We checked the effect of COE on cellular physiology such as cell proliferation assay. COE-treated cells showed reduced viability compared to control in all three cell lines used. However, the treatment of the chemotherapeutic drug, DOX was more effective compared to the COE-independent treatment. As expected, COE co-treatment with DOX was more potent compared to COE-independent treatment. However, the effectiveness of COE+DOX treatment was in a similar pattern compared to DOX-independent treatment. Figure 30.

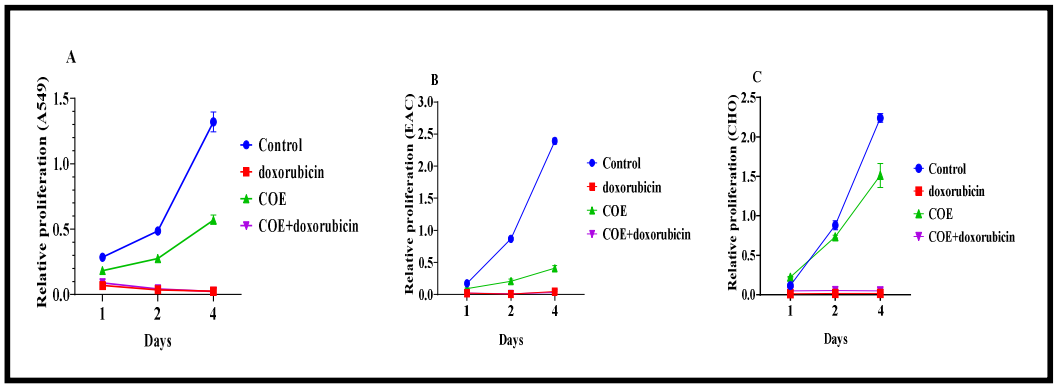


Figure 30: Effect of DOX and COE treatment on relative cell proliferation in (a) A549, (b) EAC, and (c) CHO cell lines.

5.10.3. Chemosensitivity assay

The chemosensitivity assay result shows that there was alike run in percentage viability in the control group. In COE and DOX independent treatment, there was a decrease in the percentage viability in A549, EAC and CHO cell lines. However, It was observed that the DOX-independent treatment was more potent compared to COE independent treatment. In addition, within the lower concentration of DOX and COE treatment, the percentage viability was reduced in all the cell lines compared to the rest of the treatments i.e. A549 (log concentration ~ 0.5 µg/mL), EAC (log concentration ~ 2 µg/mL), and CHO (log concentration ~ 8 µg/mL); Figure 31.

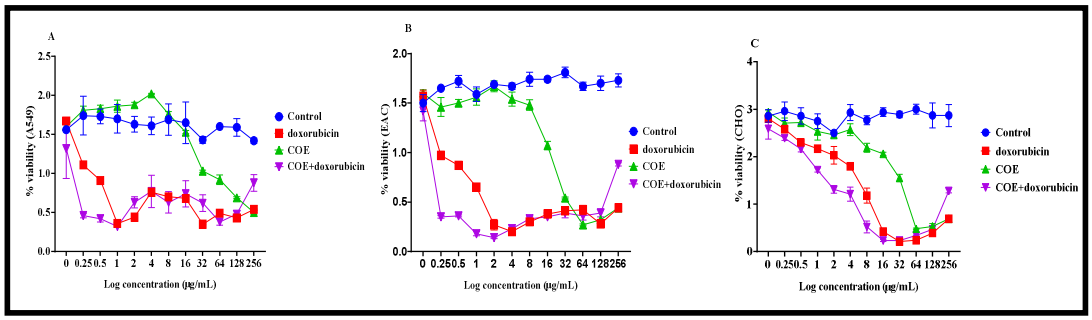


Figure 31: Effect of DOX and COE treatment on chemosensitivity assay in (a) A549, (b) EAC, and (c) CHO cell lines

5.10.4. Wound healing assay

The wound-healing assay was performed to check the effect of COE on migration. In A549, EAC, and CHO cell lines, generated wound size (at 0 hours) started decreasing gradually over time in the control group. However, as expected, DOX treatment leads to cell death and resulted in relatively higher wound size-at 12 and 24 hours compared to the control. The COE treatment showed higher wound size i.e. the reduction in migration with time compared to control which suggests that it may have a role in slowing down the cancer progression. As expected, COE co-treatment with lesser DOX concentration showed more reduction in migration. CHO cell line wound closure was much faster than the other two cell lines which allowed us to collect data for up to 12 hours only.

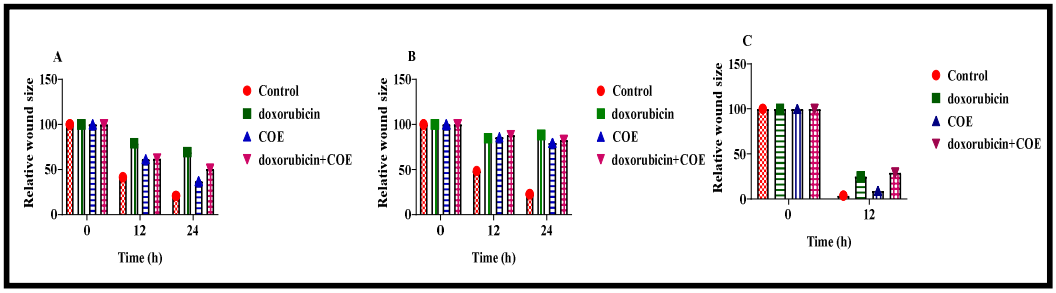


Figure 32: Effect of DOX and COE treatment on relative wound healing in (a) A549, (b) EAC, and (c) CHO cell lines.

5.10.5. Colony formation assay

In colony formation assay resulted in reduced colony formation capability up on COE treatment compared to control (the number of colonies was much higher in control *vs* COE treatment for A549 (77 vs 31), EAC (58 vs 13) and CHO (80 vs 49) cells, respectively). However, the treatment of doxorubicin alone or in combination with COE, resulted in complete inhibition of colony formation capability which is due to the lethal effect of doxorubicin.

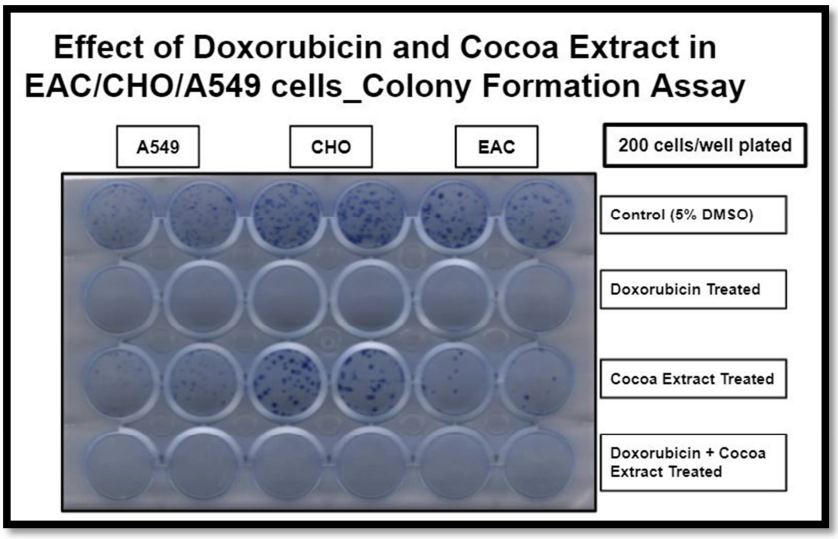


Figure 33: Colony formation assay

5.11. In vivo pharmacology

5.11.1. Ehrlich ascites carcinoma model

General observation

Mice in the EAC group showed loss of motor activity, decreased appetite, gas, and thick and bloody ascitic fluid compared to mice in the other intervention group. However, the improvement was high in the COE group, moderate in the COE + DOX group and low in the DOX group. Animals in the DOX group have also been observed to develop thin fur, red exudate eyes, watery stools, and signs of necrosis at the DOX injection site.

Figure 34: General observation

5.11.2. Survival time

To determine the effect of COE and COE + DOX on increasing survival time, treatment was carried out for day 28days and mice were observed for the entire experiment (60 days) With a pellet diet and ad libitum access to water. No deaths were observed in the normal group up to 60 days and in the EAC-treated animals; the mean survival time (MST) was 21 days. In the DOX group, MST was 25 days and the increase in life expectancy (ILS) was 19.04%. Similarly in the COE group, MST was extended to 33 days and the % ILS was 57.14%. In the COE + DOX group of animals, MST was 28 days and the % ILs was found to be 33.33% compared to the group with EAC alone.

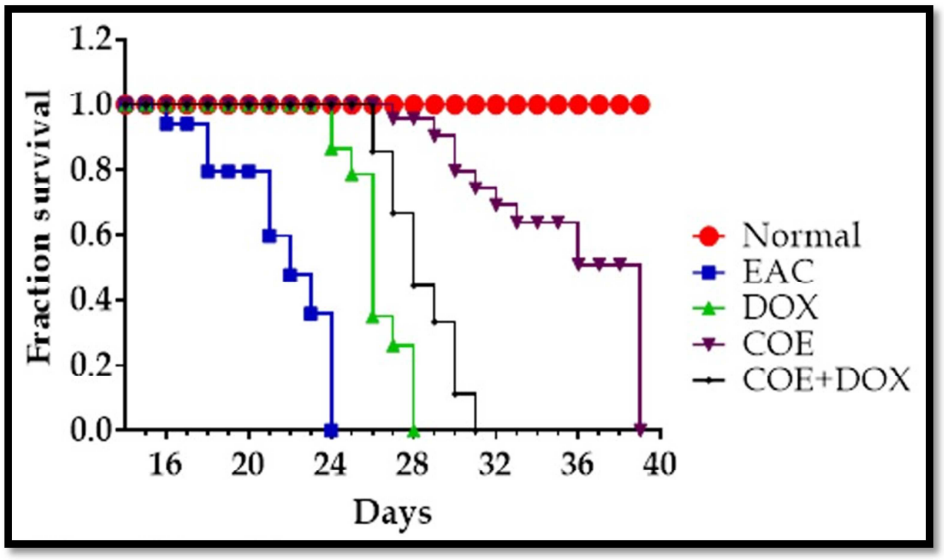


Figure 35: Survival time in EAC mice

There was a significant difference (p=0.0003; p<0.001) in Mantel-Cox log-rank in between the groups with 21.31 $\chi 2$; also, the Gehan-Breslow-Wilcoxon test reflected a significant difference (p=0.0019; p<0.01) in between the groups with 17.01 χ^2; Figure 1 represents the survival time in EAC mice.

5.11.3. Electrocardiogram

Mice in the EAC group showed a significant decrease ($p < 0.001$) in heart rate compared with the normal group, which was Significantly reversed in the COE ($p < 0.05$) and pretreated groups ($p < 0.01$) when compared with EAC. Furthermore, there was a significant increase ($p < 0.001$) in the QRS complex in the EAC group compared with the normal group. No significant change was observed in the QRS complex in any of the groups except the Pt + COE + DOX group ($p < 0.05$) compared to the EAC group. However, an observable decrease in the amplitude of the QRS complex was recorded in the COE-treated group compared with the EAC and DOX groups. Figure 36

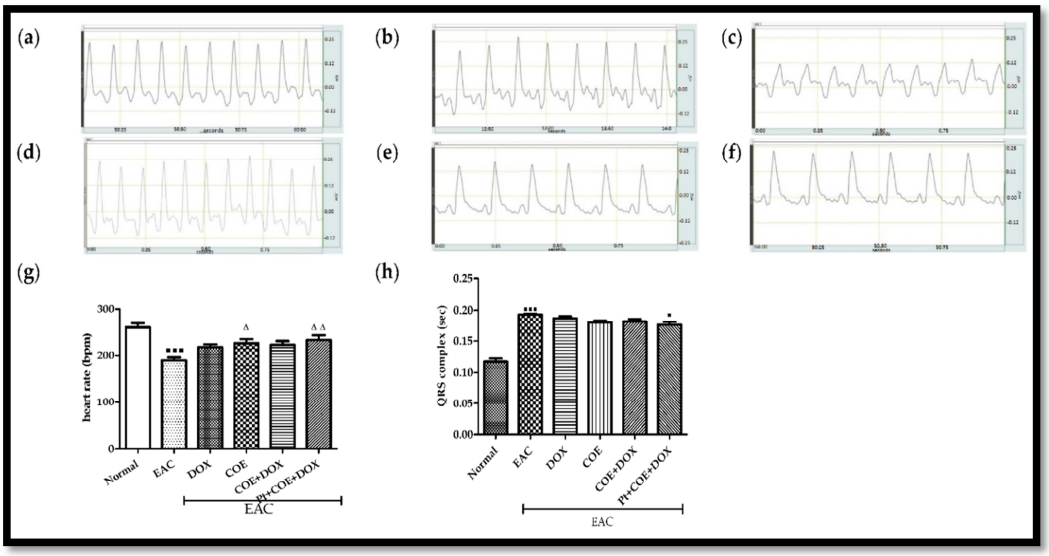


Figure 36: Effect of COE on ECG on doxorubicin-induced cardiomyopathy in EAC-induced carcinoma. Qualitative analysis of ECG of all the experimental groups; (a) Normal, (b) EAC, (c) DOX, (d) COE, (e) COE + DOX, (f) Pt + COE + DOX, (g) effect on heart rate, (h) effect on QRS complex. All values are expressed as the mean ± SEM (n = 6). Data were analysed using a one-way analysis of variance (ANOVA) followed by Tukey's test. ■■■ $p < 0.001$, compared with normal; Δ $p < 0.05$, ΔΔ $p < 0.01$, compared with EAC.

5.11.4. Physical parameters, ascitic fluid volume, and % change in body weight

A significant reduction in ascitic fluid volume ($p < 0.001$) was noted in the COE and COE + DOX groups compared with the EAC group. In addition, the percentage change in body weight in the EAC group was significantly ($p < 0.001$) higher than normal; whereas in the COE ($p < 0.001$), DOX ($p < 0.05$), COE + DOX ($p < 0.001$) groups and the pre-treatment group ($p < 0.001$), they were significantly inversely proportional to the EAC group. In addition, pre-treatment with COE showed a significant reduction ($p < 0.01$) in the percentage change in body weight compared to the alone-DOX treatment group. Figure 37

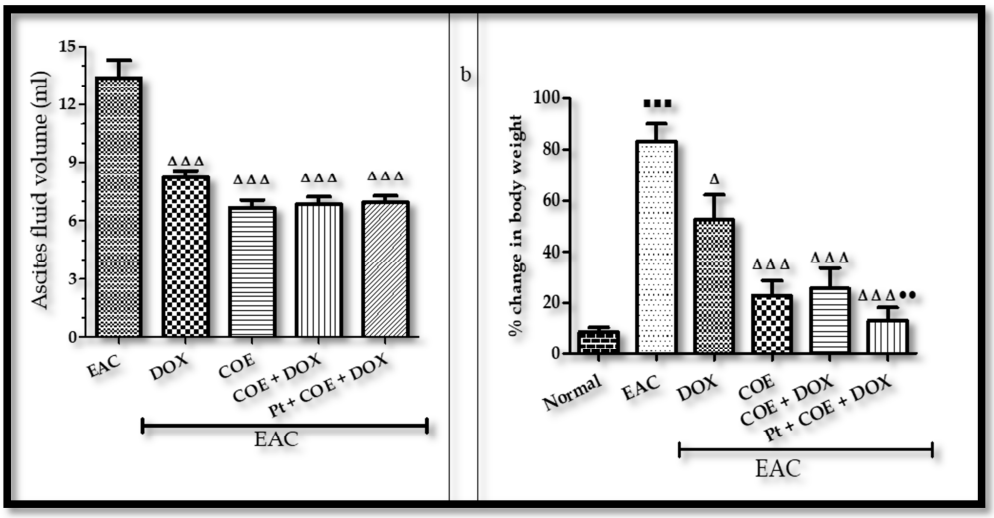


Figure 37: Ascitic fluid volume and % change in body weight.

All values are expressed as a mean ± SEM (n=6), One Way Analysis of Variance (ANOVA) followed by Tukey test ▪▪▪ $p<0.001$, compared with Normal; $^{\Delta}$ $p<0.05$, $^{\Delta\Delta}$ $^{\Delta}$ $p<0.001$, compared with EAC; •• $p<0.01$ compared with DOX.

5.11.5. Haematology

There was a significant decrease ($p < 0.001$) in red blood cells, Hb and PCV, while an observable reduction in platelet, lymphocyte and MCH levels was observed in the EAC group compared with the normal. The same blood parameters remained significantly increased ($p < 0.05, 0.01, 0.001$) in mice treated with COE, DOX alone or in combination. In contrast, there was a significant increase ($p < 0.001$) in MCHC, and WBC and an observable increase in eosinophils and monocytes were observed in the EAC group compared with the normal. In addition, the levels of these blood markers were significantly altered ($p < 0.05, 0.01, 0.001$, respectively) towards the normal value in COE and DOX-treated animals as well as in combination. Furthermore, the maximum improvement in ameliorating the haematological parameters occurred in COE-pretreated mice.

Table. 10. Hematological parameters

Groups	Normal	EAC	DOX	COE	COE+DOX	Pt+COE+DOX
RBC (cell/cmm)	9.745±0.719	5.640±0.147■■■	7.668±0.117▲▲	7.487±0.228▲	6.667±0.397	7.240±0.191▲
Hb (g%)	14.35±0.851	7.867±0.210■■■	9.53±0.387	11.37±0.705▲▲▲	10.32±0.234▲	11.03±0.255▲▲
Platelet count (cell/cmm)	14.85±2.373	9.670±0.609	17.58±1.840▲▲	11.38±0.368	12.85±0.699	14.46±0.795
PCV (%)	63.33±1.553	38.75±1.79■■■	31.98±1.677▲	35.58±1.864	34.25±1.404	37.18±0.700
MCV (fl)	60.72±2.585	53.68±0.743	50.97±1.861	51.00±1.022	50.83±0.784	52.02±2.161
MCHC (gm/dl)	25.07±0.507	28.48±0.231■■■	30.37±0.364▲▲	30.87±0.269▲▲▲	30.57±0.172▲▲▲	30.88±0.188▲▲▲
WBCs (cell/cmm)	19.81±3.502	63.32±10.0■■■	20.50±2.997▲▲▲	25.71±3.587▲▲▲	20.95±2.527▲▲▲	21.62±1.410▲▲▲
Lymphocytes (%)	88±1.238	82±3.406	83.50±3.575	83±4.619	73±0.966	68.33±0.882▲••
Neutrophils (%)	7.833±0.8333	15.67±1.406■■	10.50±1.893	7.333±0.919▲▲	12.17±0.872	13.67±2.028
Eosinophils (%)	1.167±0.4014	2.±1.000	1.667±0.333	1.333±0.210	1.±0.2582	1.±0.2582
Monocytes (%)	1.500±0.223	5.000±1.342	1.000±0.0▲▲	2.500±0.562	2.167±0.477▲	1.667±0.333▲
MCH(pg)	15.28±0.393	14.25±0.081	16.15±0.474▲▲	15.58±0.339	15.68±0.119▲	15.43±0.360

All values are expressed as a mean ± SEM (n=6), One Way Analysis of Variance (ANOVA) followed by the Tukey test.■ ■p<0.01, ■ ■ ■p<0.001, compared with Normal.▲ p<0.05, ▲▲ p<0.01, ▲▲▲ p<0.001, compared with EAC; ••p<0.01, compared with DOX.

5.11.6. Cardiac CPK-MB and LDH levels

A significant increase in cardiac CPK-MB levels ($p < 0.05$) was observed in the EAC compared to the normal animal. The mice in the DOX group showed (slightly) increased levels of CPK-MB compared to the EAC group. In addition, the COE- group showed a decrease ($p < 0.05$) in CPK-MB levels; while the COE + DOX and Pt + COE + DOX groups showed a (slight) increase in CPK-MB levels compared to the EAC group. A significantly increase ($p < 0.001$) in LDH levels was observed in the EAC group compared to normal. DOX treatment showed a significant increase ($p < 0.001$) in LDH levels compared to the EAC group. Treatment with COE, COE + DOX and Pt + COE + DOX showed a significant decrease in LDH levels ($p < 0.001$) compared to the EAC group. The LDH levels in the group treated with COE, COE+DOX and Pt+COE+DOX were closest to the normal values. Notably, the LDH level in the COE + DOX group and pre-treatment was significantly reduced ($p < 0.001$) compared to the DOX group. It was also observed that prophylactic and curative treatment with COE alone or with COE + DOX resulted in a significant improvement ($p < 0.001$) in CK-MB and LDH values are summarized in Table.11.

Table 11. Cardiac markers

Groups	CPK-MB (U/L)	LDH(U/L)
Normal	183.8±9.94	4933 ± 1573
EAC	380.2±64.18$^{\blacksquare}$	17843 ± 1718$^{\blacksquare\blacksquare\blacksquare}$
DOX	467.2±36.50	26717 ± 1301$^{\blacktriangle\blacktriangle\blacktriangle}$
COE	223.3±8.78$^{\blacktriangle}$	7125 ± 696.3$^{\blacktriangle\blacktriangle\blacktriangle}$
COE+DOX	402.5±22.03	7550 ± 874.7$^{\blacktriangle\blacktriangle\blacktriangle\bullet\bullet\bullet}$
Pt+COE+DOX	386.0±25.43	6910 ± 600.6$^{\blacktriangle\blacktriangle\blacktriangle\bullet\bullet\bullet}$

All values are expressed as a mean ± SEM (n=6), One Way Analysis of Variance (ANOVA) followed by the Tukey test.$^{\blacksquare}$ $p<0.05$, $^{\blacksquare\blacksquare\blacksquare}$ $p<0.001$, compared with Normal.$^{\blacktriangle}$ $p<0.05$, $^{\blacktriangle\blacktriangle\blacktriangle}$ $p<0.001$, compared with EAC; $^{\bullet\bullet\bullet}$ $p<0.001$, compared with DOX.

5.11.7. Hepatic enzymes

Significant increases ($p < 0.001$) in the level of ALP, AST and ALT were observed in the EAC compared to the normal. However, the DOX group showed a significant reduction in ALP ($p < 0.001$), AST ($p < 0.05$) and ALT ($p < 0.001$) compared to the EAC group. The COE + DOX and Pt + COE + DOX groups showed a significant decrease in ALP, AST and ALT levels ($p<0.001$) compared to EAC, while only AST and ALT decreased compared to DOX. The COE group also showed a significant decrease in ALP, AST and ALT values ($p<0.001$) compared to the EAC. The results are summarized in Table.12.

Table 12: Hepatic markers

Groups	ALP(U/L)	AST(U/L)	ALT(U/L)
Normal	20.83 ± 0.401	201 ± 4.69	75.5 ± 2.291
EAC	82.17 ± 4.622■■■	1196 ± 10.11■■■	264 ± 1.915■■■
DOX	31.17 ± 1.447▲▲▲	1070 ± 46.56▲	184.8 ± 2.12▲▲▲
COE	22.33 ± 0.557▲▲▲	553.8 ± 22.53▲▲▲	110.3 ± 0.802▲▲▲
COE+DOX	27.5 ± 1.784 ▲▲▲	885.8 ± 13.67 ▲▲▲•••	134.2 ± 1.276▲▲▲•••
Pt+COE+DOX	27.67 ± 1.054▲▲▲	814.2 ± 27.26▲▲▲•••	118.8 ± 1.493▲▲▲•••

All values are expressed as a mean ± SEM (n=6), One Way Analysis of Variance (ANOVA) followed by the Tukey test.■ ■ ■$p<0.001$, compared with Normal; ▲ $p<0.05$, ▲▲▲ $p<0.001$, compared with EAC; ••• $p<0.001$, compared with DOX.

5.11.8. Kidney markers

Significant ($p<0.001$) improvement in Creatinine and BUN levels was observed in the EAC when compared with the normal animals. However, these levels were significantly reversed ($p<0.001$) in all the treatment groups. Also, there was a significant decrease in BUN level observed in COE + DOX ($p<0.05$) and COE pre-treated ($p<0.001$) when compared to the DOX group. However, there was an observable difference in creatinine levels in COE + DOX and Pt + COE + DOX groups when compared to the DOX group. The results are summarized in Table.13.

Table 13: Kidney markers

Groups	Creatinine (mgs %)	BUN (mgs %)
Normal	0.125 ± 0.01	30.83 ± 0.980
EAC	0.333 ± 0.02■■■	79.83 ± 0.477■■■
DOX	0.233 ± 0.02▲▲▲	53.17 ± 0.654▲▲▲
COE	0.183 ± 0.01▲▲▲	34.5 ± 1.147▲▲▲
COE+DOX	0.2 ± 0▲▲▲	49.33 ± 0.333▲▲▲•
Pt+COE+DOX	0.191 ± 0.008▲▲▲	39 ± 0.577▲▲▲•••

All values are expressed as a mean ± SEM (n=6), One Way Analysis of Variance (ANOVA) followed by Tukey test.■ ■ ■ p<0.001, compared with Normal; ▲▲▲ p<0.001, compared with EAC; • p<0.05, ••• p<0.001, compared with DOX.

5.11.9. Lipid profile.

Significant (p<0.001) decrease in HDL values in the EAC was observed compared to mice in the normal group. The DOX-treated group also showed a decrease in HDL levels compared to the EAC group. However, HDL level was improved in COE, COE + DOX, and Pt+COE + DOX groups. LDL level significantly decreased in DOX (p<0.01), COE (p<0.05), and COE + DOX (p<0.05) groups. Cholesterol levels significantly increased in the DOX (p<0.01) and significantly decreased in COE (p<0.001) group when compared to the EAC. Results are summarized in Table 14.

Table 14: Lipid profile

Groups	Normal	EAC	DOX	COE	COE+DOX	Pt+COE+DOX
HDL (mgs %)	62.85 ± 6.419	24.00 ± 1.949$^{■■■}$	23.33 ± 1.202	43.50 ± 0.806$^{▲▲}$	30.50 ± 0.670	33.50± 0.763
LDL (mgs %)	42.33± 1.229	48.67± 2.552	36.83± 1.014$^{▲▲}$	38.67± 2.704$^{▲}$	38.17± 2.587$^{▲}$	39.67± 2.39
Cholesterol (mgs %)	84.83± 2.088	94.50± 4.272	115.0± 2.582$^{▲▲}$	70.00± 2.033$^{▲▲▲}$	89.83± 5.747$^{•••}$	88.17± 3.60$^{•••}$
Triglycerides (mgs %)	42.50± 0.885	74.00± 7.870$^{■■}$	75.50± 11.69	23.50±2.630$^{▲▲▲}$	72.33± 2.603	69.50± 4.653

All values are expressed as a mean ± SEM (n=6), One Way Analysis of Variance (ANOVA) followed by Tukey test. $^{■■}$p<0.01, $^{■■■}$p<0.001, compared with Normal; $^{▲}$ p<0.05, $^{▲▲}$ p<0.01, $^{▲▲▲}$ p<0.001, compared to EAC; $^{•••}$ p<0.001, compared with DOX.

5.11.10. Effect of COE on Enzymatic and non-enzymatic antioxidant biomarkers in heart homogenate

Significant increase in LPO (p<0.001) level was observed along with a significant decrease in GSH (p<0.001), SOD (p<0.001), CAT (p<0.001), and total thiols (p<0.01) in the EAC when compared the normal. These levels of these markers were significantly reversed in all the treatment groups (p<0.01, p<0.001) when compared to the EAC. However, in the DOX group, the GSH level was found to be non-significantly different from the EAC group. Interestingly, a significant decrease in LPO (p<0.05, p<0.05) and a significant increase in SOD (p<0.01, 0.05) and total thiol (ns, p<0.05) levels were observed in COE + DOX and Pt+COE + DOX groups, respectively compared to DOX group. Further, there was an increase in GSH levels in

the COE+DOX group and Pt+COE+DOX group ($p<0.05$) when compared to the DOX group. Results are summarized in Table. 15.

Table 15: Enzymatic and non-enzymatic antioxidant biomarkers in heart homogenate

Groups	LPO (Nano Moles/mg of protein)	GSH (µMol/mg protein)	SOD(Units/mg of Protein)	CAT (Units/mg of Protein)	Total thiol (µMol/mg of protein)
Normal	196±19.1	18.05±2.4	462.5±12.0	0.79±0.6	22.26±3.2
EAC	508±17.6$^{■■■}$	13.15±0.2$^{■■■}$	182.6±13.2$^{■■■}$	0.06±0.001$^{■■■}$	11.43±0.4$^{■■}$
DOX	218.2±18.5$^{▲▲▲}$	15.07±0.8	306.1±15.9$^{▲▲}$	0.45±0.02$^{▲▲▲}$	29.22±1.4$^{▲▲▲}$
COE	215.4±8.1$^{▲▲▲}$	24.99±2.1$^{▲▲▲}$	521.2±14.3 $^{▲▲▲}$	0.65±0.07$^{▲▲▲}$	38.44±2.7$^{▲▲▲}$
COE+DOX	148.5±7.7$^{▲▲▲•}$	20.26±0.5$^{▲▲}$	436.1±23.9$^{▲▲▲••}$	0.56±0.01$^{▲▲▲}$	35.15±0.9$^{▲▲▲•}$
Pt+COE+DOX	149.9±11.2$^{▲▲▲•}$	22.02±1.0$^{▲▲▲•}$	413.7±34.7$^{▲▲▲•}$	0.55±0.05$^{▲▲▲}$	38.7±0.8$^{▲▲▲•}$

All values are expressed as a mean ± SEM (n=6), One Way Analysis of Variance (ANOVA) followed by the Tukey test$^{■■■}$ $p<0.001$, compared with Normal; $^{▲▲}$ $p<0.01$, $^{▲▲▲}$ $p<0.001$, compared with EAC; $^{•}$ $p<0.05$, $^{••}$$p<0.01$ compared with DOX.

5.11.11. Effect of COE on Enzymatic and non-enzymatic antioxidant biomarkers in hepatic homogenate

Significantly higher LPO level ($p<0.001$) and a considerable drop in GSH ($p<0.001$), SOD ($p<0.001$), CAT ($p<0.001$), and total thiol ($p<0.01$) levels was observed in the EAC when compared to the normal mice. The levels of these markers were significantly ($p<0.001$) improved in COE, COE + DOX, and Pt + COE + DOX groups compared to the EAC group. A significant decrease in LPO level ($p<0.001$) and an increase in GSH (ns), SOD ($p<0.001$), CAT ($p<0.001$), and total thiol ($p<0.05$) levels were observed in the DOX group when compared to EAC group. Further, in COE + DOX and Pt + COE + DOX groups, there were significant decreases in LPO (ns, $p<0.05$) and increases in SOD (ns, $p<0.05$) and total thiol ($p<0.001$, $p<0.01$) levels, respectively in comparison with the mice in DOX group. Results are summarized in Table. 16.

Table 16: Enzymatic and non-enzymatic antioxidant biomarkers in the liver homogenate

Groups	LPO (Nano Moles/mg of protein)	GSH (µMol/mg protein)	SOD (Units/mg of Protein)	CAT (Units/mg of Protein)	Total thiol (µMol/mg of protein)
Normal	193.3± 14.3	32.50±0.6	427.5±16.3	0.98±0.18	142.1±6.7
EAC	649.0±57.3$^{■}$	10.07±0.3$^{■}$	156.9±19.2$^{■}$	0.14±0.05$^{■■}$	19.67±1.8$^{■}$
DOX	368.8±49.9$^{▲▲▲}$	22.47±1.1	389.4±21.2$^{▲▲▲}$	1.27±0.15$^{▲▲▲}$	37.75±5.7$^{▲}$
COE	364.7±32.5$^{▲▲▲}$	39.8±1.7$^{▲▲▲}$	595.8±27.9$^{▲▲▲}$	1.20±0.15$^{▲▲}$	115.3±3.7$^{▲▲▲}$
COE+DOX	308.3±16.5$^{▲▲▲}$	33.51±2.4$^{▲▲▲}$	428.3±07.5$^{▲▲▲}$	0.90±0.19$^{▲▲▲}$	95.22±1.3$^{▲▲▲•••}$
Pt+COE+DOX	210.0±7.2$^{▲▲▲•}$	36.49±1.5$^{▲▲▲}$	495.7±26.5$^{▲▲▲•}$	1.32±0.12$^{▲▲▲}$	77.41±2.2$^{▲▲▲••}$

All values are expressed as a mean ± SEM (n=6), One Way Analysis of Variance (ANOVA) followed by the Tukey test.$^{■}$p<0.05, $^{■■}$p<0.01, $^{■■■}$p<0.001, compared with Normal; $^{▲}$ p<0.05, $^{▲▲}$ p<0.01, $^{▲▲▲}$ p<0.001, compared with EAC; $^{•}$ p<0.05, $^{••}$p<0.01, $^{•••}$ p<0.001, compared with DOX.

5.11.12. Effect of COE on Enzymatic and non-enzymatic antioxidant biomarkers in kidney homogenate

A Significantly increase ($p<0.001$) in LPO level and a significant decrease in GSH ($p<0.001$), SOD ($p<0.001$), CAT ($p<0.001$), and total thiols ($p<0.001$) were observed in the EAC group in comparison with the normal group of animals. A Significantly reduction in LPO level ($p<0.001$) was observed in all treatment groups compared to the EAC group. Further, in COE, COE + DOX, and Pt + COE + DOX groups, a significant increase in GSH ($p<0.001$, 0.001, 0.001), SOD ($p<0.001$, 0.05, 0.01), CAT ($p<0.001$, 0.05, 0.05), and total thiols ($p<0.01$, ns, 0.05), respectively was observed when compared to animals in the EAC group. Additionally, it was noted that GSH levels ($p<0.001$, 0.001) in the COE + DOX and Pt + COE + DOX groups significantly increased as compared to the DOX group. The results are summarized in Table.18.

Table 17: Enzymatic and non-enzymatic antioxidant biomarkers in kidney homogenate

Groups	LPO (nM/mg of protein)	GSH (µM/mg protein)	SOD (U/ mg of protein)	CAT (U/ mg of protein)	Total thiol (µM/mg of protein)
Normal	214.2±31.1	21.6± 0.3	496.6±64.6	0.75±0.12	68.85±10.6
EAC	439.3± 24.8$^{■■■}$	12.29±0.3$^{■■■}$	182.5±5.7$^{■■■}$	0.14±0.05$^{■■■}$	25.45± 2$^{■■■}$
DOX	227.3±9.704$^{▲▲▲}$	12.28±0.4	314.9±26.	0.47±0.06$^{▲}$	30.49±2.5
COE	207.9±8.1$^{▲▲▲}$	23.37±0.7$^{▲▲▲}$	490.7±23.6$^{▲▲▲}$	0.57±0.07$^{▲▲}$	63.19±5.3$^{▲▲}$
COE+DOX	213.8±17.1$^{▲▲▲}$	21.38±0.2$^{▲▲▲•••}$	364.7±31.2$^{▲}$	0.49±0.06$^{▲}$	44.19± 3.9
Pt+COE+DOX	222.9±17.7$^{▲▲▲}$	22.69±0.6$^{▲▲▲•••}$	430.2±46.8$^{▲▲}$	0.53± 0.05$^{▲}$	55.17±6.3$^{▲}$

All values are expressed as a mean ± SEM (n=6), One Way Analysis of Variance (ANOVA) followed by Tukey test $^{■■■}$p<0.001, compared with Normal; $^{▲}$ p<0.05, $^{▲▲}$ p<0.01, $^{▲▲▲}$ p<0.001, compared with EAC; $^{•••}$ p<0.001, compared with DOX.

5.11.13. Histopathology

Histopathology of heart

Cardiac tissue in the normal animals revealed normal morphology of myocardial cells with complete and orderly myocardial fibres and normal myocardial interstitial (Figure 38a). However, myocardial cells in the animals of the EAC group and DOX group showed inflammation, widened intermuscular plane, with broken and disorganized myocardial fibres (Figure 38 b and c.). However, these features were attenuated in the COE, COE + DOX, and pre-treatment groups when compared to EAC and DOX groups (Figure 38 d - f.). Similarly, congestion was found significantly decreased in all treated groups compared to the EAC group (Figure 38g). Oedema was observed only in EAC and DOX alone treatment groups. These results suggest that COE administration alone or in COE + DOX ameliorates cardiac remodelling.

The bar graph shows the scoring of the pathological changes (Figure 38).

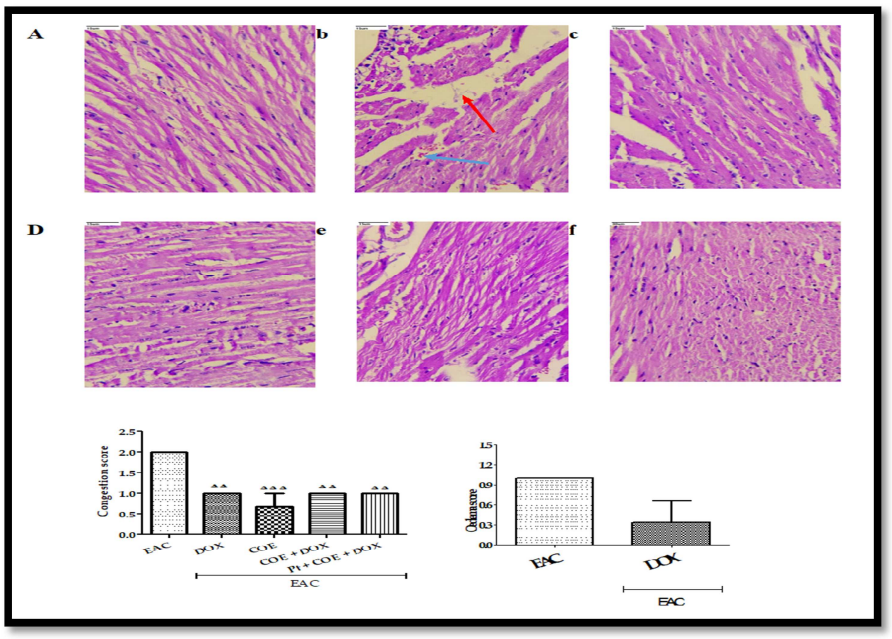


Figure 38: Histopathology of heart

Photograph of heart section of different treatment groups stained with Haemotoxylin and Eosin. Plates at 40x magnification a: Normal, b: EAC, c: DOX, d: COE, e: COE+DOX and f: Pt+COE+DOX.

EAC group (b) shows oedema (red arrow) and congestion (blue arrow).

All values are expressed as a mean ± SEM (n=3), One Way Analysis of Variance (ANOVA) followed by Tukey test $^{\Delta\Delta}$ $p<0.01$, $^{\Delta\Delta\Delta}$ $p<0.001$ compared with EAC.

5.11.14. Histopathology of liver

The H&E-stained liver sections of normal mice showed normal hepatic tissue architectures. The EAC group and DOX alone group showed spotty necrosis, inflammation, and venous congestion area in hepatic cells (Figure 39b and c). However, COE, COE + DOX, and Pt + COE + DOX groups showed improved regeneration of hepatic tissue with a lesser degree of inflammation, sinusoidal congestion, venous congestion, and Kupffer cell hyperplasia in the hepatic section (Figure 39 d -f). No spotty necrosis was seen in the COE alone group of animals and a noticeable decrease in spotty necrosis was found in animals of COE + DOX and Pt + COE + DOX groups. Further, there was a significant decrease in venous congestion in COE alone and Pt + COE + DOX ($p<0.05$) and in COE + DOX ($p<0.01$) compared to the EAC group. No significant improvement was found in DOX alone group. Similarly, sinusoidal congestion was significantly improved in the COE + DOX and Pt + COE + DOX ($p<0.05$) group of animals. The bar graph shows the scoring of the pathological changes. Figure 39

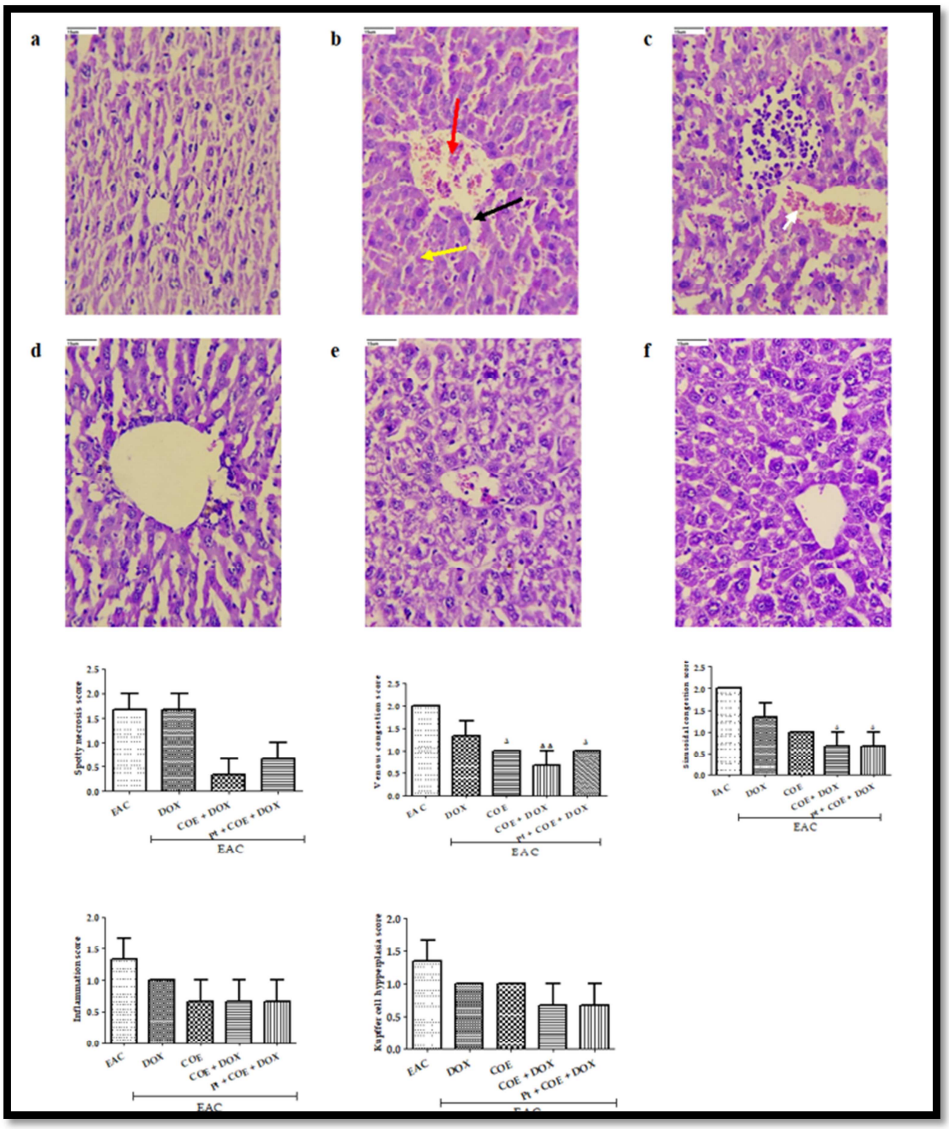


Figure 39: Histopathology of the liver

Photograph of Liver section of different treatment groups stained with Haemotoxylin and Eosin. Plates at 40x magnification a: Normal, b: EAC, c: DOX, d: COE, e: COE+DOX and f: Pt+COE+DOX.

EAC group (b) shows Central vein congestion (red arrow),Venous Congestion (black arrow), Inflammation (yellow arrow) and Dox group (c) showing Sinusoidal Congestion(white arrow).

All values are expressed as a mean ± SEM (n=3), One Way Analysis of Variance (ANOVA) followed by Tukey test.$^{\Delta}$ P<0.05, $^{\Delta\,\Delta}$ p<0.01, compared with EAC.

5.11.15. Histopathology of kidney

The histopathological examination of kidney tissue showed an observable decrease in glomerular congestion, tubular congestion, cytoplasmic vacuoles, and peritubular inflammation within all treatment groups compared to animals in the EAC group. The bar graph shows the scoring of the pathological changes in Figure 40.

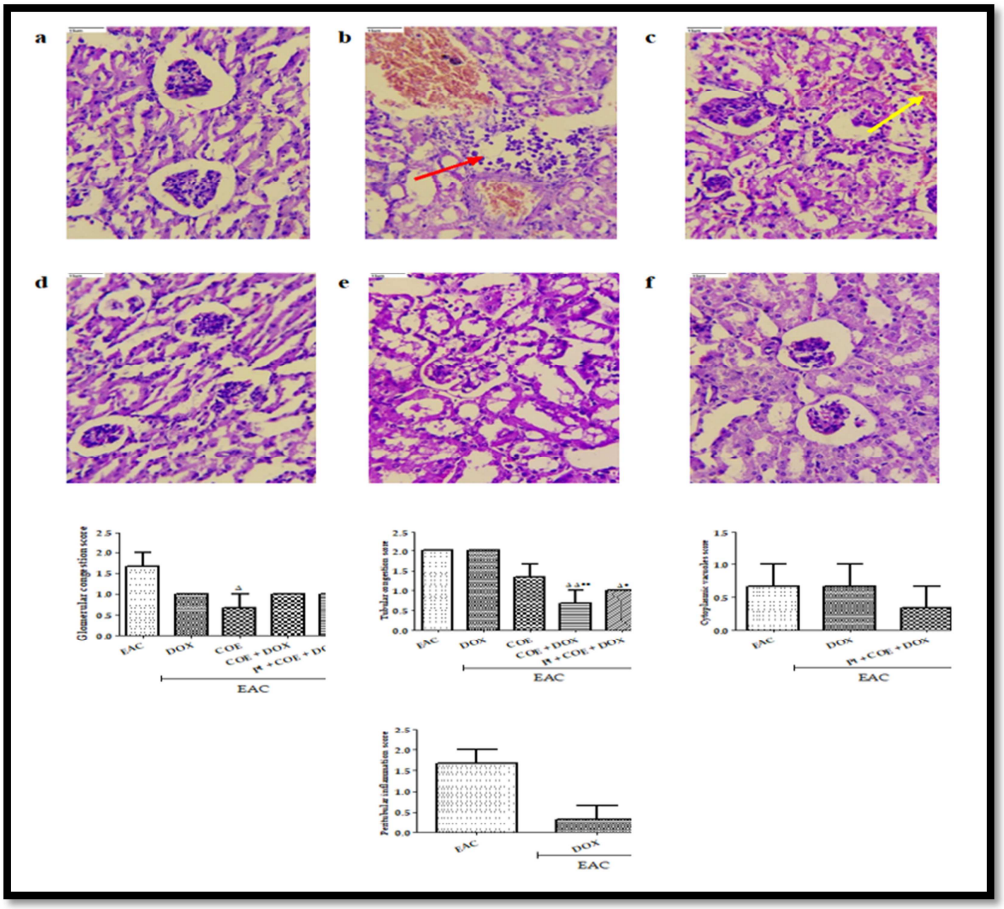


Figure 40: Histopathology of kidney: Photograph of kidney sections of different treatment groups stained with haematoxylin and eosin. Plates at 40× magnification.

(a) Normal, (b) EAC, (c) DOX, (d) COE, (e) COE + DOX, (f) Pt +COE + DOX, (g) Glomerular congestion score, (h) Tubular congestion, (i) Cytoplasmic vacuoles score, (j) Peritubular inflammation score. EAC group (b) shows peritubular inflammation (red arrow) and DOXox group (c) shows tubular congestion (yellow arrow).

All values are expressed as the mean ± SEM (n = 3). Data were analysed using one-way analysis of variance (ANOVA) followed by Tukey's test. Δ $p < 0.05$, ΔΔ $p < 0.01$, compared with EAC; ● $p < 0.05$, ●● $p < 0.01$, compared with DOX**.**

5.11.16. Ehrlich ascites carcinoma solid tumor model

5.11.17. Effect of COE on the survival

There was no death was observed in the normal group throughout the study. However, deaths were observed from the 28^{th} to the 39^{th} day in the group EAC. Further, COE and DOX treatment has shown an increased life span, and it was maximum within the COE200+DOX group. (Figure 41) Log-rank (Mantel-Cox) Test reflected the increased life span of COE-treated animals in combination with DOX compared to the rest of the animals with χ^2=185.8, and Df = 4.

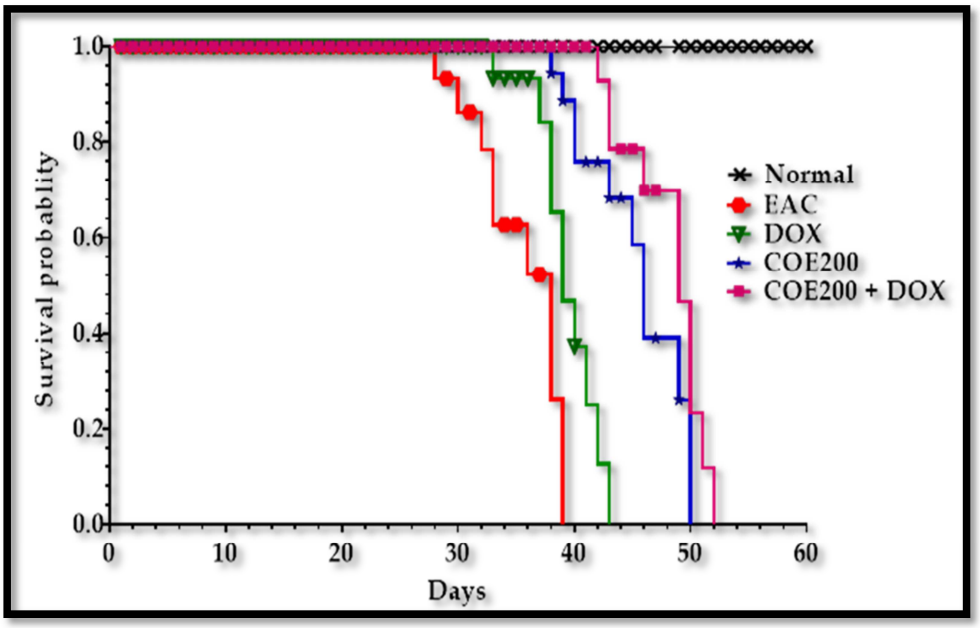
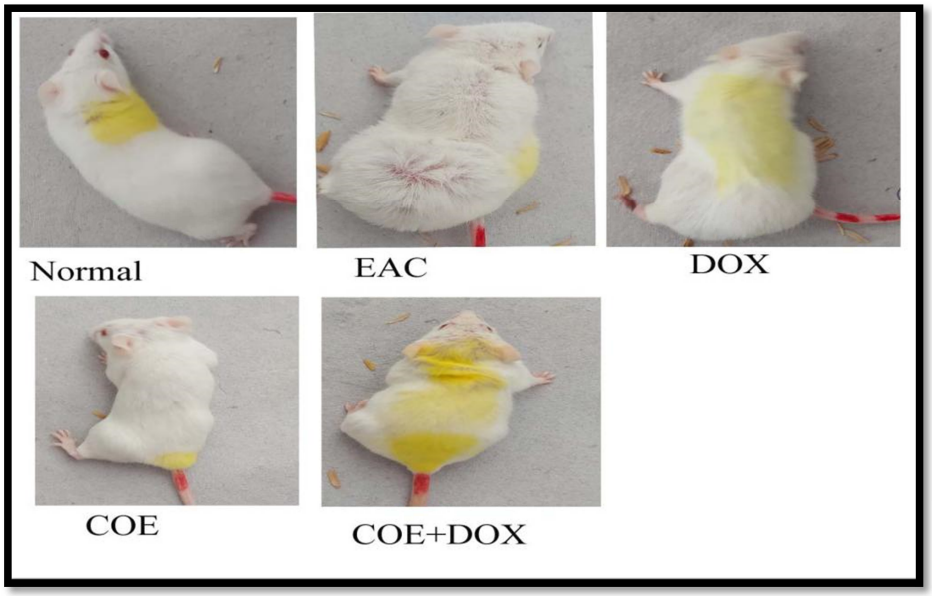


Figure 41: Kaplan–Meier survival curve for COE and DOX-treated groups



5.11.17. COE's impact on body weight, tumor weight and size.

There was a significant increase in percentage change in body weight ($p<0.001$) within EAC compared to normal which was significantly reversed in all treatment groups (Figure 42a). In addition, the combined action of COE and doxorubicin significantly decreased the change in body weight compared to

doxorubicin alone. Additionally, there was a significant decrease ($p<0.001$) in tumor weight with DOX and COE independent and combination treatment. Further, the combined action of COE and DOX Significant decreased tumor weight compared to DOX group. (Figure 42b) Likewise, there was a significant decrease ($p<0.001$) in the progression of tumor size was observed from the 14th day of treatment with COE treatment groups. Further, on the 21st day, there was a significant decrease ($p<0.001$) in tumor size with all treatment groups compared to EAC, although the combination group showed a significant decrease ($p<0.01$) in the tumor size compared to the independent DOX group. Figure 43

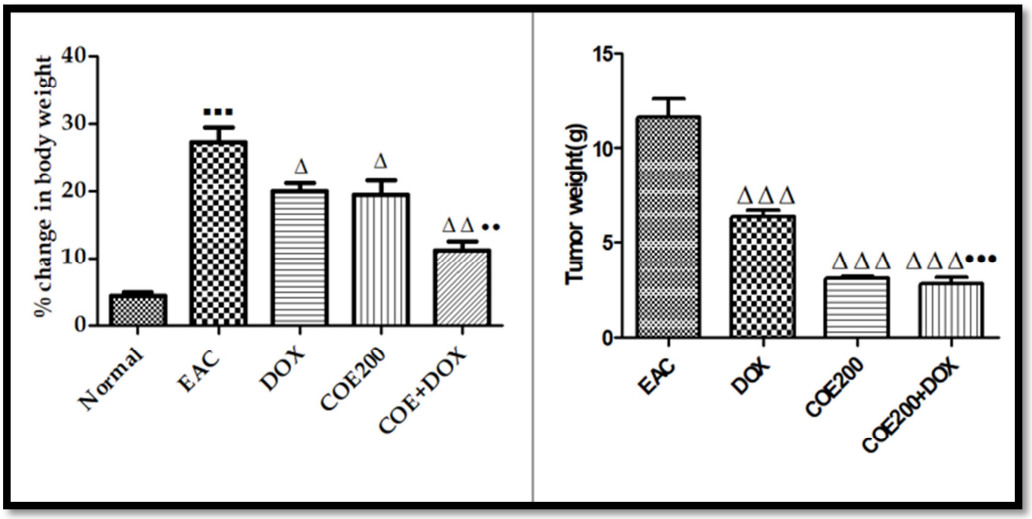


Figure 42: (a) % change in body weight and (b)tumor weight (g) in solid Ehrlich tumor-bearing mice after 21 days of treatment.

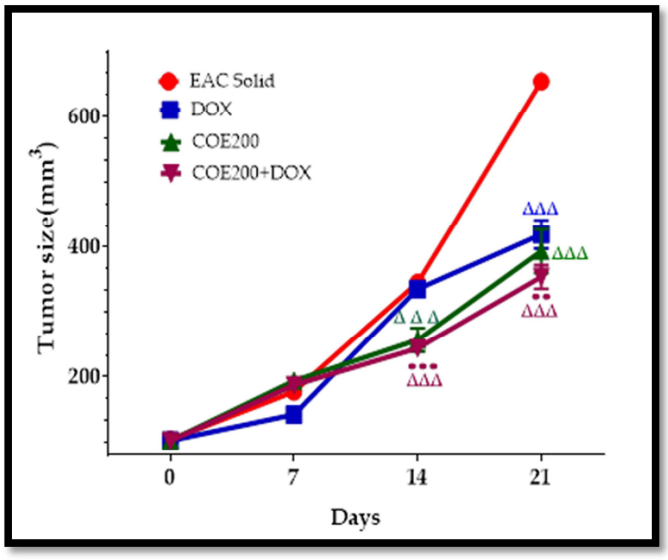


Figure 43: Tumor volume (mm3) in solid Ehrlich tumor bearing mice

All values are expressed as a mean ± SEM (n=6), One Way Analysis of Variance (ANOVA) followed by Tukey test ■■■p<0.001, compared with Normal; $^{\Delta}$ p<0.05, $^{\Delta\Delta}$ $^{\Delta}$ p<0.001, compared with EAC; ••p<0.01 compared with DOX.

5.11.18. Effect on haematological parameters.

There was a significant decrease in haemoglobin (p<0.001) in EAC (9.2±0.15%) compared to normal (13.83±0.68%) which was significantly increased (p<0.01) with COE-independent treatment. It was also observed that a further decrease in haemoglobin in the DOX group (7.9±0.18%) was significantly increased in the combination group (10.77±0.20%) compared to DOX. Further, there was a significant increase in WBC count (p<0.001, 51.02±1.5 cell/cm) in EAC compared to normal (27.21±2.1 cell/cm). Which was significantly decreased (p<0.001) in all treatment groups compared to EAC Table.16. In addition, there was a significant decrease in RBC and Platelet count seen in EAC (p<0.001) these cell count was further decreased after DOX treatment. The reduction in the cell count was reversed in COE treatment groups. There was a significant decrease (p<0.001) in, PCV,

MCHC and lymphocytes compared to normal were noted to be significantly improved ($p<0.005$-0.001) with DOX and COE independent and in combination. In contrast, there was a significant increase ($p<0.001$) in neutrophil eosinophil monocytes and MCH ($p<0.01$) within EAC compared to normal which was ameliorated with independent or combined action with DOX and COE Table 19.

Table. 18. Haematological parameters

Groups	Normal	EAC	DOX	COE200	COE200+DOX
Hb (% g)	13.83 ±0.68	9.2 ±0.15■■■	7.9 ± 0.18	11.33± 0.13▲▲	10.77± 0.20•••
WBCs (cell/cmm)	27.21 ± 2.1	51.02 ±1.5■■■	25.56±1.7▲▲▲	33.91 ±0.1▲▲▲	29.77 ± 1.4▲▲▲
RBC million (cell/cmm)	8.79 ± 0.2	4.995 ±0.2■■■	4.23 ± 0.2	7.397 ±0.0▲▲▲	6.03 ± 0.2▲•••
Platelet count (cell/cmm)	1030 ± 20.9	789 ±20■■■	645.6±16.2▲▲	1007 ± 23.8▲▲▲	870.4 ± 35.6•••
PCV (%)	54.78 ± 1.4	27.37 ±2.2■■■	32.23 ± 1.0	36.97 ± 0.7▲▲▲	39.63 ±0.4▲▲▲••
MCV (fl)	51.52 ± 0.8	52.4 ±0.2	51.43 ± 0.30	50.37 ± 0.06▲	51.37 ± 0.2
MCHC (gm/dL)	31.75 ± 0.7	24.12 ±0.7■■■	31.18±0.3▲▲▲	32.03 ± 0.4▲▲▲	32.05 ± 0.5▲▲▲
Lymphocytes (%)	74.5 ± 0.6	11.67 ±1.05■■■	19.67±0.6▲▲▲	32 ± 1.0▲▲▲	44.5± 1.5▲▲▲•••
Neutrophils (%)	21.83 ± 0.8	80.83 ±1.6■■■	63 ± 0.4▲▲▲	59.83 ± 0.9▲▲▲	54.17± 1.7▲▲▲•••
Eosinophils (%)	0.66 ± 0.2	2.5 ± 0.2■■■	1.5 ± 0.2▲	1.8 ± 0.1	1.3 ± 0.2▲▲

Monocytes (%)	0.3 ± 0.2	5.16 ± 0.7 ■■■	2.5 ± 0.4 ▲▲	3.16 ± 0.1 ▲	2.16 ± 0.4 ▲▲
MCH(pg)	16.45 ± 0.2	17.9 ± 0.1 ■■	17.1 ± 0.2	15.52 ±0.3 ▲▲▲	15.77 ± 0.3 ▲▲▲••

All values are expressed as a mean ± SEM (n=6), One Way Analysis of Variance (ANOVA) followed by the Tukey test. ■ ■ $p<0.01$, ■ ■ ■ $p<0.001$, compared with Normal. ▲ $p<0.05$, ▲▲ $p<0.01$, ▲▲▲ $p<0.001$, compared with EAC; •• $p<0.01$, ••• $p<0.001$compared with DOX.

5.11.19. Effect of COE on Cardiac, Hepatic and Kidney Biomarkers

In the present study, we observed a significant increase ($p<0.001$) in CK-MB, LDH, ALT, AST, ALP, creatinine, and BUN levels within EAC groups. Further, these biomarkers were significantly increased ($p<0.05$-0.001) in the DOX group. In contrast, these markers were significantly ($p<0.05$-0.001) ameliorated in the COE group and combination group. Likewise in combination, a significant decrease in these parameters was observed compared to DOX.

Table 19: Effect of COE treatment on Cardiac, Hepatic and Kidney Biomarkers

Treatment	CK-MB(U/L)	LDH (U/L)	AST (U/L)	ALT (U/L)	ALP (U/L)	Creatinine (mgs %)	BUN (mgs %)
Normal	170.2±1.6	1955±14.6	217.7±16.3	75.5 ± 2.2	20.83±0.4	0.125±0.0	30.83±0.9
EAC	251.5±2.4$^{■■■}$	4268±22.5$^{■■■}$	620.3±21.2$^{■■■}$	150.7±2.9$^{■■■}$	37.83±2.6$^{■■■}$	0.28 ±0.0$^{■■■}$	77.33±0.9$^{■■■}$
DOX	360±2.6$^{▲▲▲}$	5264±20.8$^{▲▲▲}$	898.7 ± 25.4$^{▲▲▲}$	234.8 ± 5.8$^{▲▲▲}$	46.67±1.0$^{▲▲}$	0.32±0.0$^{▲}$	87.67 ±1.6$^{▲▲▲}$
COE200	236.8±1.5$^{▲▲}$	2654±15.5$^{▲▲▲}$	512.2 ± 17.2$^{▲▲}$	80 ± 6.7$^{▲▲▲}$	29.33±1.1$^{▲▲}$	0.23 ±0.0$^{▲▲}$	55.67 ±0.9$^{▲▲▲}$
COE200+ DOX	255.5±3.2$^{•••}$	4176±18.3$^{▲•••}$	547.7 ± 7.9 $^{▲▲•••}$	124.5±1.4$^{▲▲•••}$	39.5±2.0$^{•}$	0.2383±0.0$^{▲•••}$	70.17±1.64$^{▲▲•••}$

All values are expressed as a mean ± SEM (n=6), One Way Analysis of Variance (ANOVA) followed by Tukey test $^{■■■}$p<0.001, compared with Normal; $^{▲}$ p<0.05, $^{▲▲}$ p<0.01, $^{▲▲▲}$ p<0.001, compared with EAC; $^{•}$ p<0.05, $^{••}$p<0.01, $^{•••}$ p<0.001, compared with DOX.

5.11.20. COE on antioxidant biomarkers

There was a significant increase in LPO level (p<0.001) in the heart, liver, and kidney within EAC compared to normal, which was significantly reversed with COE treatment. Further, there was a significant increase in LPO (p<0.001) in the DOX group compared to EAC. Which was reversed with combination treatment. Similarly, there was a significant decrease (p<0.01, 0.001) in cardiac, hepatic, and kidney GSH, SOD, and CAT activities compared to normal. Doxorubicin-independent treatment had no significant influence over GSH, SOD, and CAT activities in the heart, liver, and kidney. However, COE treatment groups significantly ameliorated (p<0.05-0.001) their GSH, SOD, and CAT activity in the heart, liver, and kidney compared to EAC. In addition, the combined action of COE with doxorubicin significantly

($p<0.05$-0.001)/observable influenced GSH level, SOD, and CAT activity in the heart, liver, and kidney.

Table 20: Effect of administration of COE on DOX-induced depletion of, LPO, GSH, SOD and CAT levels in heart, liver and kidney tissues of mice.

Tissue	Treatment	LPO(Nano Moles/mg of protein)	GSH(µMol/mg protein)	SOD(Units/mg of Protein)	CAT(Units/mg of Protein)
Heart	Normal	50.77 ± 1.7	26.29 ± 3.1	196.1 ± 2.7	0.57 ± 0.05
	EAC	177.3 ± 4.1 ■■■	17.51 ± 1.1 ■■	76.87 ± 3.7 ■■■	0.39 ± 0.02 ■■
	DOX	229 ± 3.08 ▲▲▲	14.99 ± 0.7	77.07 ± 5.9	0.31 ± 0.02
	COE200	148.3 ± 3.3 ▲▲▲	25.56 ± 1.05 ▲	111.5 ± 7.2 ▲▲	0.55 ± 0.01 ▲
	COE200+DOX	163.6 ± 1.9 ▲•••	22.75 ± 0.8 •	105.4 ± 9.0 ▲•	0.49 ± 0.02 ••
Liver	Normal	86.51 ± 6.5	164.2 ± 3.4	192.6 ± 11.2	0.5 ± 0.06
	EAC	165.8 ± 7.1 ■■■	91.47 ± 4.9 ■■■	127.8 ± 15.4 ■■	0.2 ± 0.02 ■■
	DOX	212 ± 7.6 ▲▲▲	88.39 ± 2	124.8 ± 9.3	0.1 ± 0.03
	COE200	105.8 ± 4.1 ▲▲▲	149.5 ± 9 ▲▲▲	181.9 ± 3.4 ▲	0.4 ± 0.03 ▲
	COE200+DOX	168.5 ± 3.4 •••	131.7 ± 3.3 ▲▲▲•••	176.2 ± 7.6 ▲•	0.3 ± 0.05
Kidney	Normal	252.9 ± 6.7	29.7 ± 2.4	46 ± 3.7	0.79 ± 0.02
	EAC	526.9 ± 18.9 ■■■	13.03 ± 1.6 ■■■	22.5 ± 2 ■■■	0.38 ± 0.01 ■■■
	DOX	483.1 ± 15.2 ▲▲▲	12.2 ± 0.4	14.9 ± 0.3	0.16 ± 0.01 ▲▲▲
	COE200	238 ± 13.2 ▲▲▲	26.2 ± 1.7 ▲▲▲	36.81 ± 2.8 ▲▲	0.60 ± 0.03 ▲▲▲
	COE200+DOX	303.3 ± 11.9 ▲▲▲	20.41±0.8 ▲▲▲•••	21.7 ± 0.9	0.42 ± 0.0 •••

All values are expressed as a mean ± SEM (n=6), One Way Analysis of Variance (ANOVA) followed by Tukey test ■■■p<0.001, compared with Normal; ▲ p<0.05, ▲▲ p<0.01, ▲▲▲ p<0.001, compared with EAC; •p<0.05, ••p<0.01, •••p<0.001, compared with DOX. LPO: lipid peroxidation, GSH: glutathione, SOD: superoxide dismutase, CAT: catalase.

5.11.21. Effect of COE on cardiac, hepatic, kidney, and solid tumor histology.

The normal architecture was observed in the heart, liver, and kidney tissue in the normal group). Further, in the EAC group, congestion and myofibrillar degeneration were noted in cardiac tissue (Figure 44). Venous and sinusoidal congestion, kupffer cell hyperplasia, spotty necrosis, apoptosis, inflammation, and hepatocellular dysplasia were noted within hepatic tissue(Figure 45), followed by tubular and glomerular congestion, glomerular atrophy, tubular cell swelling, inflammation, widening of the bowmen space, and cytoplasmic vacuoles were observed in kidney tissue (Figure 46). In addition, these variables were traced in the EAC group, which were markedly raised with independent doxorubicin treatment. Further, these histopathological changes were mitigated in COE-treated groups. We also observed the tumor tissue which showed a maximum number of cords and nests of tumor cells, congestion, angiogenesis, haemorrhage, invading muscle infiltration, fibrosis, necrosis, anaplasia, and mitotic activity in the EAC group compared to the rest of the interventions.Figure47

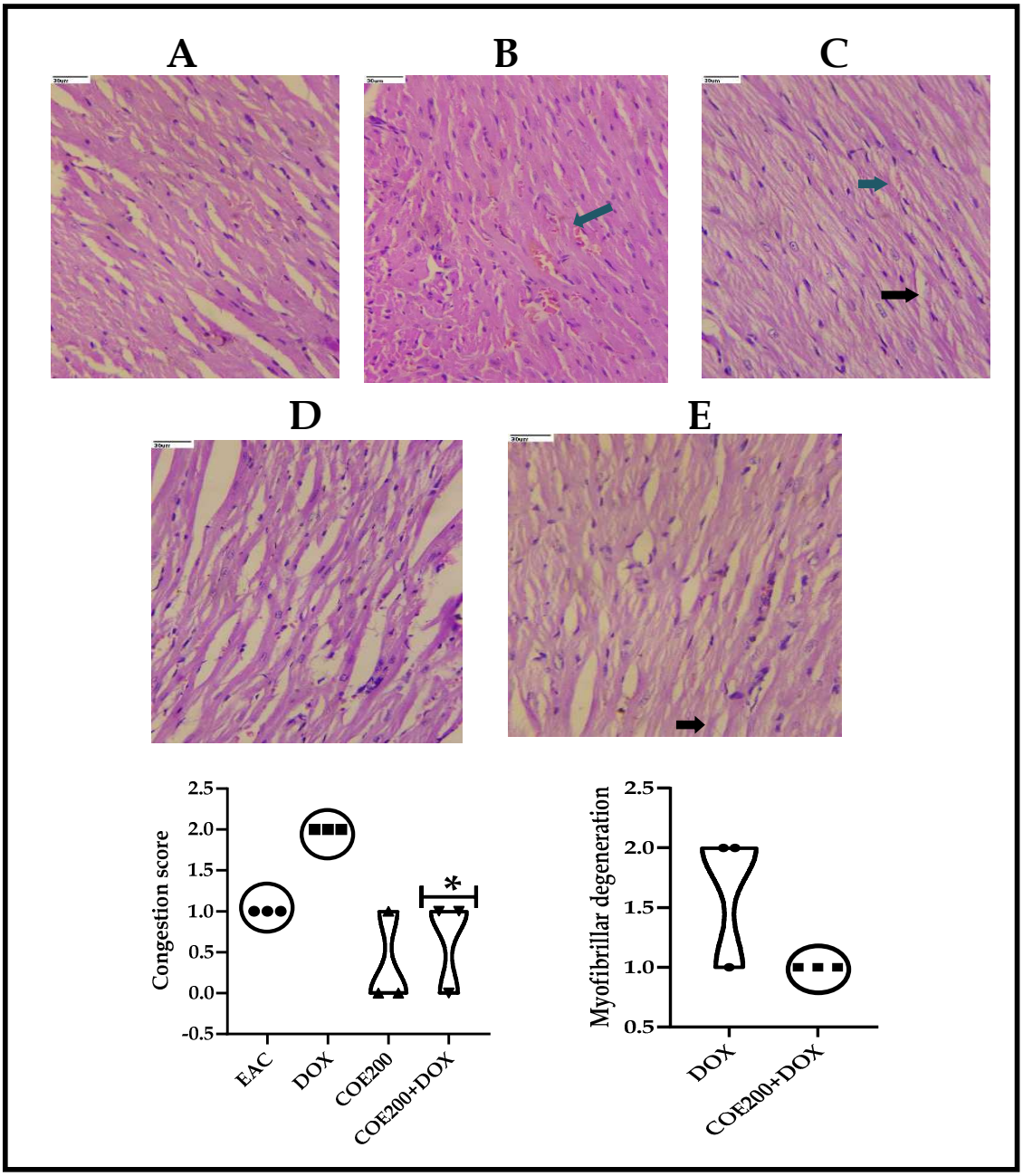


Figure 44: Histopathology of heart. Photograph of heart section of different treatment groups stained with Haemotoxylin and Eosin. Plates at 40x magnification a: Normal, b: EAC, c: DOX, d: COE, e: COE+DOX. EAC group (b) shows congestion (blue arrow) and myofibrillar degeneration (black arrow). All values are expressed as a mean ± SEM (n=3), One Way Analysis of Variance (ANOVA) followed by Tukey test ● $p<0.05$, compared with DOX.

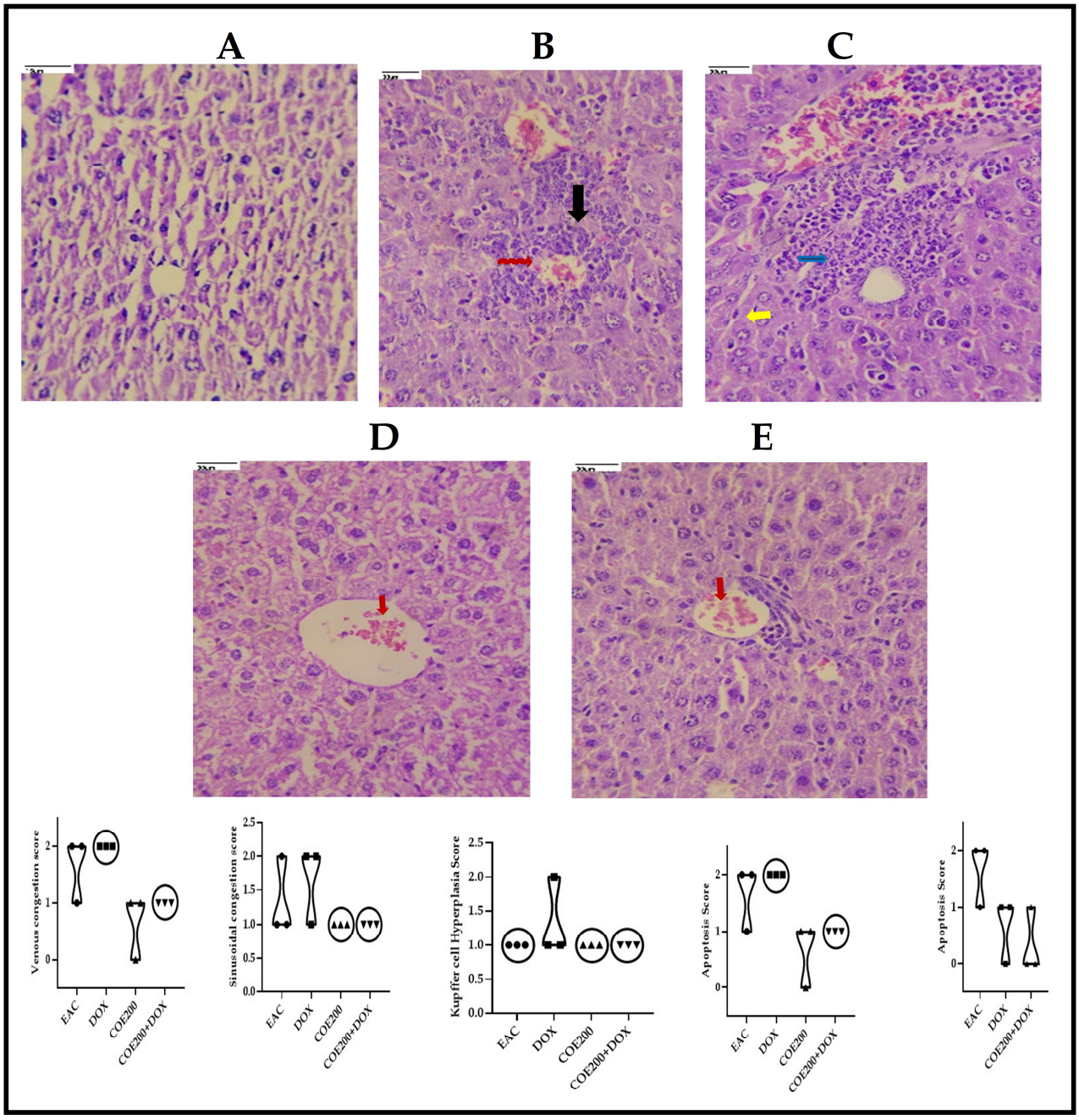


Figure 45: Histopathology of the liver. Photograph of liver section of different treatment groups stained with Haemotoxylin and Eosin. Plates at 40x magnification a: Normal, b: EAC, c: DOX, d: COE, e: COE+DOX. EAC and DOX groups are showing venous & sinusoidal congestion (red and blue), Kupffer cell hyperplasia (yellow), apoptosis, and spotty necrosis. (black). All values are expressed as a mean ± SEM (n=3), One Way Analysis of Variance (ANOVA) followed by Tukey test.

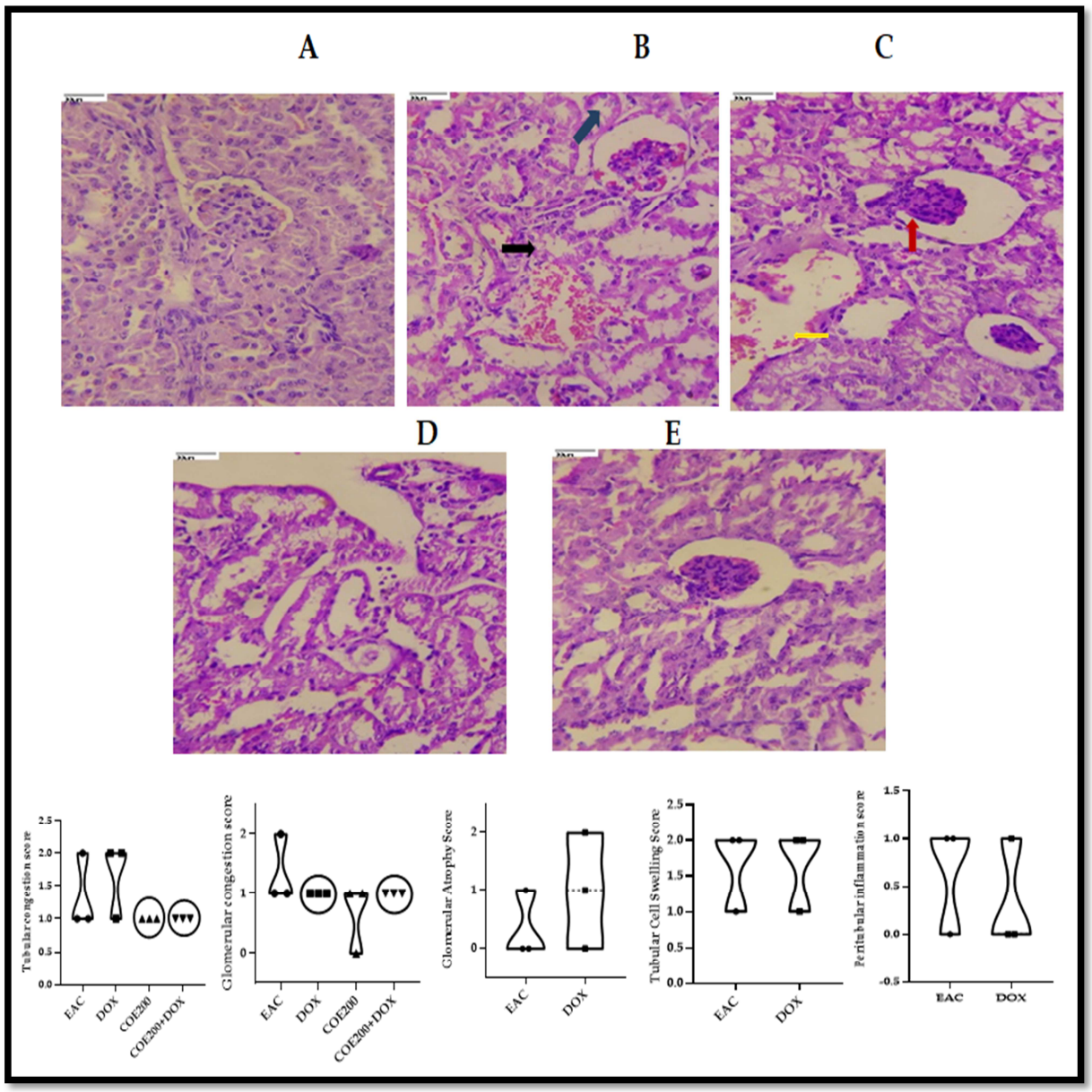


Figure 46: Histopathology of kidney. Photograph of kidney section of different treatment groups stained with Haemotoxylin and Eosin. Plates at 40x magnification a: Normal, b: EAC, c: DOX, d: COE, e: COE+DOX. EAC and DOX groups are showing Tubular (black) & glomerular (blue) congestion, Inflammation, glomerular atrophy,(red) and tubular cell swelling(yellow). All values are expressed as a mean ± SEM (n=3), One Way Analysis of Variance (ANOVA) followed by the Tukey test.

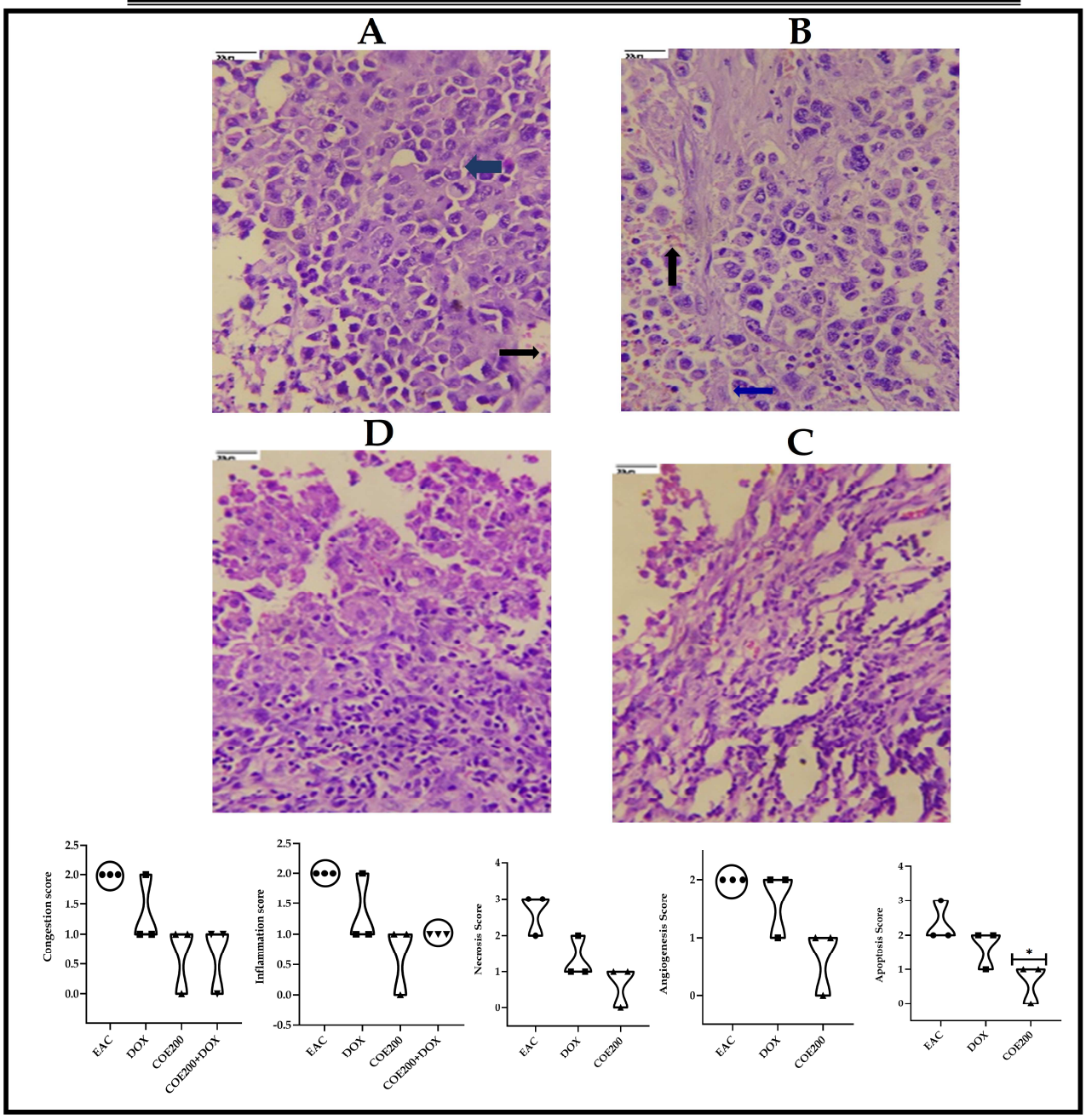


Figure 47: Histopathology of Tumor. Photograph of tumor section of different treatment groups stained with Haemotoxylin and Eosin. Plates at 40x magnification a: EAC, b: DOX, c: COE, d: COE+DOX. EAC and DOX groups are showing congestion (black), Inflammation (blue) Hemorrhage, Angiogenesis, apoptosis, and Necrosis (red). All values are expressed as a mean ± SEM (n=3), One Way Analysis of Variance (ANOVA) followed by Tukey test ▲ $p<0.05$ compared with EAC.

6. DISCUSSION

In the present study, the protective effect of *Theobroma cacao* bean against doxorubicin-induced organ toxicities in EAC-bearing mice *via in silico, in vitro,* and *in vivo* studies. The effects of COE on the heart, liver, and kidneys were assessed. Treatment with COE was found to minimize the doxorubicin toxicities on the vital organs; evidenced by significant improvement of the above-mentioned organs' biomarkers in animals with COE+DOX treatment. COE reduced the organ toxicity associated with doxorubicin and also acted as an anticancer agent promoted the anticancer activity of doxorubicin. Further, *in vitro* studies showed that COE alone has a role in the reduction of the cell proliferation rate and colony formation capability in EAC as well as human cancer cell lines A549 compared to normal cell line CHO. Also, COE enhanced doxorubicin potency in the inhibition of cancer cell proliferation and migration. Additionally, an *in silico* approach identified the probable protein targets and pathways modulated by the bioactive of cocoa against oxidative stress and cancer using the network pharmacology approaches.

Physicochemical properties of cocoa such as moisture content, ash value, extraction value, pH, and fluorescence analysis were evaluated along with phytochemical screening, HPTLC, and LCMS characterization. Evaluation of these parameters helps to ensure the quality of the test material and to identify any adulteration. The ash value provides information about the quality and purity of the crude powder and the moisture content ensures the rate of degradation and the extractive value assures the possible availability of phytochemicals in the crude powde.r[147] In the present work, all physicochemical parameters were within the range recommended in Ayurvedic Pharmacopoeia of India (API). Further, the yield of the

COE was found to be 5.8% w/w. The HPTLC fingerprints showed the presence of thiobromin (60.1 ± 2.9) mg/gm and epicatechin (50.8 ± 1.03) mg/gm in the extracts. Further, the reported compounds of the COE were traced with the LC-MS profile.

Previous reports suggest the antioxidant activity and anticancer activity of COE is due to the presence of high content of phenol and flavonoid.[148] The quantification of the total phenol content of COE was 72.5 g gallic acid equivalent /mg extract and total flavonoid content.was 165 g QE/mg extract quercetin equivalent (QE), respectively. Additionally, DPPH and nitric oxide scavenging activity showed the antioxidant potential of COE.

6.1. In silico studies

Doxorubicin has been reported to cause oxidative stress resulting in organ toxicity.[74] Hence, a set of *in silico* studies was carried out to evaluate the possible mechanism of action of COE on oxidative stress and cancer progression. The current study traced 54 documented bioactives of cocoa to propose a credible mechanism against oxidative stress and cancer pathogenesis. The bioactives were predicted to target 220 proteins and were found to be involved in 104 pathways, of which 50 targets and 21 pathways were found to be associated with oxidative stress and cancer. Among the 50 targets predicted, EGFR was identified as a major druggable target for cocoa bioactives and modulated in 15 different pathways within the compound protein pathway network. Out of 34 bioactive, 11 were identified to target EGFR. EGFR is a potent oncogene frequently overexpressed in a variety of cancers, which is a well-known therapeutic target in cancer therapy.[149] EGFR inhibitors have beneficial effects against cell proliferation and progression in a wide variety of cancer types. [150]Previously, catechins have been reported to prevent colon cancer and hepatocellular

cell carcinoma by preventing the activation of the RTKs, primarily EGFR, IGF1R, and VEGFR2, and compared pathways.[151] In addition, cocoa polyphenols exhibit potent antioxidant, anti-inflammatory, and chemo-protective effects by direct inhibition of PI3K and MEK1 activity by TNF and upregulating VEGF.[152] Further, an *in vitro* study demonstrated a combination of glucose-(-)-epigallocatechin-3- gallate derivatives against non-small cell lung cancer.[153] Epicatechin, an antioxidant flavonoid, regulates nitric oxide production and exhibits anti-inflammatory effects in addition to cardiovascular protective effects on vascular endothelium.[89] Therefore, network analysis and predicted affinity of bioactives from cocoa towards EGFR seem to be in concurrence with these findings and provide possible molecular modes of action of cocoa as a potential anti-cancer nutraceutical. In addition, hirsutrin (galactoside of quercetin) showed stable complex formation with EGFR during the MD simulation. Similarly, the hyperosid formed eight interactions with EGFR, of which five interactions were with active site residues. A previous study by Kern *et al.,*[154] identified hirsutrin(7.5 μM) and hyperoside (6.7 μM) as EGFR inhibitors. Moreover, we compared the obtained data concerning a known EGFR inhibitor i.e. erlotinib, which scored binding energy of - 7.0 kcal/mol and formed two hydrogen bond interactions with active site residues.

In the network analysis, the arachidonic acid metabolism pathway, cAMP, Rap1, Ras, Phospholipase D, cGMP-PKG, MAPK, PI3K-Akt, and VEGF signalling pathways were found to be highly enriched pathways modulating multiple protein molecules. A previous study demonstrated flavonoids from cocoa to exhibit potent anti-inflammatory effects through modulation of the arachidonic acid pathway by the inhibiting 5-lipoxygenase enzyme (ALOX5). [155] Similarly, in the current study, flavonoids were identified to disrupt the arachidonic acid pathway by targeting 7 key

proteins (AKR1C3, ALOX12, ALOX5, CYP2C9, PLA2G10, PLA2G2A, and PLA2G5). The arachidonic acid metabolism pathway is an important metabolic pathway in which cytochrome P450 (CYP) monooxygenases, cyclooxygenases, lipoxygenases, and phospholipase A2 are potentially involved and play crucial roles in various pathophysiological functions *via* inflammatory response, oxidation, cell proliferation, survival, angiogenesis, invasion, and metastasis, which can promote carcinogenesis.[156] Additionally, cocoa flavonoids are known to have anti-proliferative action, cause apoptosis, and inhibit angiogenesis.[157–160] Moreover, catechin, epicatechin, quercetin, and procyanidin, as well as dimer extracts of procyanidin derivatives, are reported to down-regulate NF-κB and AP-1 in cancer cell lines.[161–164] Hence, the current study findings on the anti-cancer activity of cocoa could be due to the modulation of the arachidonic acid metabolism pathway and other intracellular signalling pathways *via* cAMP, Rap1, Ras, phospholipase D, cGMP-PKG, MAPK, PI3K-Akt, and VEGF.

The level of ROS generation in the tissue depends on the balance between oxidants and antioxidants. In endothelial cells, ROS-mediated angiogenesis *via* various stimuli *via* angiopoietin-I, angiogenin, VEGF, EGF, urotensin-II, shear stress, and hypoxia is a major contributor to cancer.[164,165] The common mechanism involved in cancer is dysregulation of the EGFR, which plays a vital role in cell survival, growth, differentiation, and tumorigenesis.[166] Further, angiogenin and VEGF are probably the most widely found initiators of angiogenesis.[165–167] VEGF activates Rac1- dependent NOX to induce ROS production, which sequentially provokes signalling pathways involved in endothelial cell proliferation, migration and anti-apoptotic cascade. [168,169]

6.2. In vitro studies

In this context, our study further aimed to investigate the *in vitro* antioxidant and cytotoxic potential of COE. The main goal of cancer chemotherapy is to target cancer cells without exhibiting toxicity to normal cells, and this is a limitation of the use of current chemotherapy agents.[170]

As a result, the lead molecule's antioxidant capacity and selective toxicity on normal and cancer cells must be considered in cancer treatment. Also, there is a close relationship between oxidative stress and the spread of cancer. Exogenous antioxidants can protect against oxidative stress and harm to DNA and proteins, which lowers the risk of cancer.[171] To prevent the growth of tumors, the use of natural antioxidants, in conjunction with the current treatment, is ideal.[172] Further, the MTT cytotoxicity assay demonstrated the higher toxicity of COE on EAC compared to CHO. The EAC cell line is a well-established cancer cell line with overexpression of EGFR and is known to be involved in oxidative stress and cancer progression. [173–175]. In contrast, the CHO cell line is a normal epithelial cell line that does not express EGFR.[176] Furthermore, COE alone has shown a role in the reduction of the cell proliferation, colony formation, and migratory potential of EAC as well as A549. The effect of COE on cancer cells has proved to be more potent than the normal one. Also, COE enhanced the doxorubicin potency when used in combination with a lower concentration of doxorubicin in the inhibition of cancer cell proliferation, and migration. The findings of scratch assay suggest that the COE might have a role in the inhibition of epithelial-mesenchymal transition (EMT), the first step of metastasis. The induction of the EMT process leads to the resistance to chemotherapeutic drugs.[177] The EMT inductions may reduce the cell proliferation rate and increase the

expression of proteins responsible for inhibiting apoptosis as well as the increased expression of transporter-compared proteins to efflux the drugs.[177] The COE might be involved in the modulation of EMT initiation. Some anti-proliferative agents have already been designed to inhibit EMT initiation.[178] The natural product COE can alternatively be used as a replacement for anti-proliferative agents. The COE may be involved in the modulation of signalling processes involved in cancer pathogenesis and progression and may enhance the chemotherapeutic efficacy of chemotherapeutic agents like doxorubicin. In cancer pathogenesis, rapid activation of the ROS system has been reported,[179] which can be neutralized by selected traditional drugs.

In this study, we found that COE had a stronger cytotoxic effect on EAC, A546cells compared to CHO. This indicated that the bioactive present in COE that have a stronger tendency to slow down the growth of tumor cells than normal cells. As evident that IC_{50} values of COE were relatively higher in normal cell lines than in tumor cells.An earlier investigation by Corcuera *et al.,*[180] also demonstrated polyphenols of cocoa as a potential antioxidant agent in HepG2 cells treated with mycotoxins ochratoxin A. and Martin *et al.*[181] reported the anti-apoptotic activity of cocoa polyphenols in tert-butyl hydroperoxide-induced cellular death and apoptosis in HepG2 cells.[165] This antiapoptotic impact was linked to decreased ROS production, avoidance of ERK deactivation and JNK activation, and prevention of caspase-3 activation. Also, numerous researchers demonstrated polyphenolic compounds as potent EGFR tyrosine kinase inhibitors.[154,182,183] Hence, the current study corroborates the previous literature for cocoa phytocompounds may have more affinity toward cancer cells to inhibit the EGFR task, which was demonstrated *via* docking studies.

Our study further provides add-on molecular and bioinformatics support to previous studies by deciphering the possible roles of action of phytocompounds from cocoa at the molecular level. The entire set of phytoconstituents and the study of all of them together along with their interactions may not be feasible, although a modest attempt has been made to draw a network to understand the systemic functions to the extent possible within the scope of this study. Our study has used "herbal informatics' a combination of knowledge on the use of botanicals in traditional medicine with modern-day bioinformatics using computational advancements, which is essentially the use of high-tech computational studies and simulations in establishing the validity of existing traditional uses through the reverse pharmacology approach.

6.3. In vivo studies

In the present study, to assess the efficacy of COE on doxorubicin-induced organ toxicities in two different murine cancer models *viz.*, Ehrlich Ascites Tumor which is directly involved with many vital organs such as heart, liver and kidney and the solid tumor model represents the benign tumor.

6.3.1. Study 1. Protective activity of COE against doxorubicin-induced toxicities in a mouse bearing an ascites tumor model.

Previously, cardiotoxic, nephrotoxic, and hepatotoxic effects of doxorubicin therapy[7,184,185] and cocoa in preventing multiple organ damage were also reported.[20,95,99] Hence, study was initiated to investigate the role of COE as a health supplement on the heart, liver, and kidney in doxorubicin-treated EAC-bearing mice. COE-independent treatment and in combination with doxorubicin in tumour-bearing mice showed a significant deceleration of cancer progression compared to doxorubicin-independent treatment. Additionally, COE alone and in combination with

doxorubicin resulted in a significant increase in the MST compared to doxorubicin independent treatment and the EAC group. These results indicate that COE did not interfere with the anticancer activity of doxorubicin; additionally, it reduced doxorubicin-induced organ toxicity and, therefore, has the potential to serve as a nutraceutical or complementary medicine.

COE treatment in combination with doxorubicin and pretreatment with the COE showed a significant decrease in the percentage change in body weight and ascites fluid volume in mice compared to the EAC. Interestingly, we observed a significant decrease in the percentage change in body weight in the COE-pretreated mice compared to DOX, suggesting the prophylactic activity of COE in cancer progression.Previous studies have reported that abnormal ECG patterns are seen during doxorubicin treatment such as increased width of the QRS complex, majorly contributing to ventricular hypertrophy, myocardial infarction, altered cardiac function, and other conduction abnormalities.[186,187] In the current study, an increase in the width of the QRS complex and a reduction in heart rate was observed in the EAC and DOX groups. However, in all COE-treated groups, abnormal ECG resulting from doxorubicin and EAC was reversed, which suggests the cardioprotective role of COE against doxorubicin and EAC-induced cardiotoxicity.[188]

Alterations in haematological parameters such as myelosuppression and anaemia in the EAC mouse model have been reported.[189] Similarly, in our present study, analogous findings were observed in EAC-bearing mice. In the treatment group, both doxorubicin and COE reversed these haematological parameters in EAC-bearing mice; better amelioration was noted in the COE group compared to DOX which suggests the beneficial effect of COE over doxorubicin treatment on

hematopoiesis. In addition, it has been reported that anaemia in EAC mice is caused by iron deficiency (hemolytic and myelopathic conditions), leading to a compromised RBC count.[190] In contrast, the RBC count in the doxorubicin and COE-treated mice was significantly increased compared to that in the EAC group, suggesting the beneficial role of COE along with doxorubicin treatment.

Elevated CPK-MB and LDH levels are considered important biomarkers of cardiac myocyte damage, especially during the clinical follow-up of doxorubicin therapy. The free radicals generated during doxorubicin chemotherapy cause considerable injury to the myocardium, which causes an increase in membrane permeability and thus the release of CPK-MB and LDH.[191] In the present study, doxorubicin treatment showed 2.54-fold and 5.41-fold increase in CPK-MB and LDH levels, respectively, compared to normal. Independent COE treatment and in combination with doxorubicin significantly reduced the CPK-MB (2.10-fold and 1.16-fold, respectively) and LDH (3.67-fold and 3.53-fold, respectively) levels, reflecting the cardioprotective activity of COE. Along with these parameters, LPO elevated levels and GSH, SOD, CAT, and total thiol decreased levels in the EAC group were substantially reversed in all COE-treated groups compared to animals treated with doxorubicin alone. This is suggestive of COE scavenging free radicals generated during cancer propagation and doxorubicin therapy, thereby rendering beneficial effects to the host.

Nayagam *et al.,*[192] reported that doxorubicin-associated cardiotoxicity is due to the accumulation of circulating free fatty acids, leading to blockage of the coronary arteries. Cocoa ameliorates the lipid profile in dyslipidaemic conditions in the complex pathogenesis of lipid and glucose metabolism.[193] These two observations

prompted us to assess the COE effect on lipid-lowering properties in EAC and doxorubicin-induced hyperlipidemia.[81,194] COE alone and in combination with doxorubicin significantly reversed the altered parameters, *viz*., HDL, TC, and TG, which reflected the shielding role of COE against hyperlipidemia, which, in turn, protect the cardiac injury and preserved cardiac function.

Hepatocyte damage due to doxorubicin-induced ROS, specifically superoxide anions, compromises mitochondrial function and aggravates liver damage.[81] Similarly, EAC cells affect the liver through the accumulation of ascetic fluid and by the leakage of liver enzymes such as ALP, AST, and ALT into the serum.[195] Thus, the enzyme content in the liver serves as a biomarker for hepatotoxicity. In addition to the liver, DOX and EAC also contributed to kidney damage. Previously, Mutar *et al.*,[196] reported that Ehrlich tumors are a reason for kidney damage by increasing urea and creatinine levels. These changes in urea and creatinine levels in the kidney contribute to the increased glomerular capillary permeability and tubular atrophy,[197] which is responsible for kidney failure. In our study, the elevation of both hepatic and renal markers in the EAC and DOX groups was observed, which was significantly reversed in COE in-dependent treatment or combination with doxorubicin. These findings support the beneficial role of COE against EAC and doxorubicin-associated toxicities and also demonstrate its vital role in regulating liver and kidney functions by balancing the serum biomarkers. Furthermore, a COE-mediated antioxidant defensive mechanism was also observed in both liver and kidney tissue. The elevated level of LPO and reduced levels of GSH, CAT, SOD, and thiols observed in both the EAC- and doxorubicin-treated groups were reversed in all CEO-treated groups, establishing a beneficial role in cells protection from ROS and reducing organ toxicities concerning the heart, liver, and kidney.

Accumulation of fluid in the intravascular and interstitial spaces in cardiac tissue results in cardiac load, leading to congestion triggered by cardiac oedema. [198] Mishra *et al.,*[188]. It is demonstrated that EAC triggers cardiac dysfunction as reflected by congestion scores. In this study, a higher congestion score in EAC mice was observed compared to other treatment groups. Furthermore, oedema was observed in the EAC-and doxorubicin-treated groups, which was countermanded in all groups with COE treatment, indicating that COE potentially repaired the cardiac damage caused by doxorubicin and EAC. Further, in liver tissue, spotty necrosis, venous congestion, sinusoidal congestion, inflammation, and Kupffer cell hyperplasia serve as indicators of hepatic tissue damage.[199,200] In the present and previous studies, similar histopathological findings have also been identified in both EAC mice.[201] and doxorubicin-treated animals.[202] However, in this study, animals treated with doxorubicin alone did not show any improvement in spotty necrosis but showed reduced venous congestion, sinusoidal congestion, and inflammation. Furthermore, the COE in all treated groups showed significant improvement in the reduction of doxorubicin and EAC-induced hepatic damage. This could be due to the accumulation of lipids and upregulated lipogenesis, as evidenced by upregulated LDL serum levels. The doxorubicin metabolite doxorubicinol is reported to upregulate the ROS system, which may disturb the homeostatic function of hepatocytes and alter multiple biological processes, cellular components, and molecular functions within it, leading to nonalcoholic fatty liver pathogenesis. In the present study, COE treatment reversed the EAC and doxorubicin-induced liver damage. This could be due to potential antioxidant activity owing to the presence of certain biomarkers, such as catechin, (-)-epigallocatechin gallate, hirsutrin, hyperoside, and cinaroside (molecules having a higher hydrogen-donating capacity).

Renal dysfunction induced by cardiac and hepatic injury leads to an increase in renal interstitial pressure on the entire capillary and tubules, which is triggered by an increase in glomerular and tubular congestion.[203] A similar renal dysfunction with elevated tubular and glomerular congestion has been reported in EAC and doxorubicin-associated toxicities.[204,205] These effects could be due to the increase in the ROS system or free radicals and the presence of toxic doxorubicin metabolites in the nephron, which needs to be further investigated. In this study, similar histopathological observations were recorded in the EAC-induced and doxorubicin-treated animals. Interestingly, this damage was reversed in the COE-treated groups, suggesting the beneficial effects of COE and nephroprotective activity during doxorubicin treatment in the carcinoma model.

In our study, the Ehrlich ascites model responded superior to COE treatment compared to doxorubicin. This may be due to the ascites microenvironment favouring oxidative stress,[204] the proliferation of tumor cells,[206] and comparatively less susceptible of Ehrlich ascites cells towards oxidative stress .[207] These factors may also contribute to the reduced chemotherapeutic potential of doxorubicin in ascites tumor models compared to COE due to its dual advantage of antioxidative and anticancer activity. In addition, COE also attenuates the adverse effects produced by doxorubicin on non-tumor cells without compromising its cancer therapeutic potential. This proves the protective effect of COE, suggesting considering it as a health supplement during cancer chemotherapy.

6.3.2. Study 2. Protective activity of COE against Doxorubicin-induced toxicities in a mouse-bearing ascites solid tumor model

In solid tumor model, COE significantly reduced the doxorubicin-induced toxicites in cardiac, hepatic, and nephritic tissues in the EAC-induced solid tumor mice model. The cell protective nature of COE towards cardiac myocytes, hepatocytes, and nephrons; manifested through the histological and biochemical investigations; could be through the termination of Fenton and redox reactions, as there were ameliorated enzymatic and non-enzymatic antioxidant biomarkers.

Combinatorial therapy of COE with doxorubicin in tumor-bearing mice showed an increase in survival time by 1.20 fold *vs* the DOX group. Within 21 days of treatment, we observed a significant decrease in tumor size in doxorubicin and COE-treated animals; the outcome was further effective if we proceed with combined treatment, which was evidenced *via* the assessment of tumor size. This decrease in tumor size with combined treatment could be the outcome of the synergetic effect, which might be due to the anti-cancer potency of COE.[208] Also, there was a significant increase in percentage change in body weight in the EAC group, which pointedly declined with the combined and independent action of both agents. In addition, there was no significant difference between the percentage change in the body weight within the doxorubicin and COE-treated groups, However, the percentage change in body weight was significantly lower in combination compared to DOX; suggests COE's synergetic effect towards doxorubicin. In addition, this decrease in percentage change in body weight supports the outcome of control tumor size with each intervention. Likewise, we observed a significant reduction in tumor weight with doxorubicin and COE independent and combination action over tumor

weight; a significant reduction in tumor weight in combination *vs* DOX; point's synergistic effect. Also, the anti-tumour effect of COE reflected in our study is the auxiliary outcome; conveyed *via* a remarkable increase in the lifespan.

In chemotherapy, doxorubicin has a chief contribution in dealing with many cancers. However, it produces severe toxic effects on hemorheological parameters, which is one of the limiting factors in implementing doxorubicin chemotherapy.[209] Herein we observed significant amelioration of multiple haematological parameters with the combined action of COE with doxorubicin *vs* alone DOX. Previously, it has been reported that doxorubicin metabolites disturb haematological function; could be doxorubicin-activated ROS system.[210]In addition, ROS has a direct impact on Hb, RBC, WBC, platelet and other haematological parameters, which directly affects cellular apoptosis due to compromising immunity, affecting the transport of nutrition and oxygen transport.[211] Previously COE has been reflected for its potent antioxidant activity due to the presence of phenolic bioactive and flavonoids (catechin, epicatechin, and procyanidins)[212,213]; this may trigger scavenging free radicals and terminate redox and Fenton reaction within the ROS system which could have driven the ameliorated haematological parameters.

In cancer chemotherapy, organ toxicities are one of the major complications and it affects the homeostatic function, which is most commonly reported in doxorubicin therapy.[214,215] Therefore, we quantified multiple parameters like CK-MB, LDH, AST, ALT, ALP creatinine and BUN in different treatment groups. We found that these biomarkers were significantly increased in doxorubicin alone treatment compared to EAC, indicating chemotherapy-induced organ toxicities. Likewise, free radical generation during doxorubicin therapy causes significant damage to the

myocardium, resulting in increased membrane permeability and the release of CPK-MB and LDH enzymes. Doxorubicin treatment showed a 1.43-fold and 1.23-fold increase in CPK_MB and LDH enzymes compared to EAC. Also, the liver and kidney showed an increased AST level by 1.44 folds, ALT by 1.55 folds, Creatinine by 1.14 folds, and BUN by 1.13 folds compared to EAC. Interestingly, these parameters were significantly reversed in the combinatorial regimen of COE along with doxorubicin, which showed the excellent activity of COE in the protection of vital organs in chemotherapy. This armed action of cocoa is owing to its defence against oxidative stress induced during chemotherapy.[216] Zieba *et al.,*[217]confirmed that cocoa in the form of dark chocolate high in flavonoids may be a good strategy for reducing cardiovascular risk by having beneficial effects in inhibiting platelet aggregation, lowering blood pressure, reducing dyslipidemia, and lowering plasma glucose levels**.** Reactive oxygen species (ROS), which are generated during drug biotransformation processes, can bind and react with cellular components in the liver to cause liver damage and thereby impair liver function**.**[218] Antioxidants present in cocoa increase nitric oxide levels, which in turn prevents lipoperoxidation and cell death and have greater hepatoprotective potential.[219] A similar trend was observed when antioxidant biomarkers were monitored. The elevated levels of LPO and decreased GSH, SOD, CAT, and total thiol in the DOX group compared to the EAC group were substantially reversed in all COE-treated groups, augmenting its effectiveness in organ protection. Similar findings were reported previously for the potency of COE in the neutralisation of the ROS system generated *via* various stress responses that could have been ameliorated as evidenced by multiple investigations by regulating the homeostatic functions of various organs.[220]

The aforementioned markers were further supported by the histopathological examination, which revealed, there was a significantly increased congestion score and myofibrillar degeneration in EAC solid tumor model, which was further increased with doxorubicin treatment. Similar findings of cardiotoxicity were reported by Reddy *et al.*,[59] The COE was shown to be cardio-protective in EAC solid tumor model. Further, within the EAC and DOX groups, we traced a remarkable rise of spotty necrosis, apoptosis, inflammation, hepatocellular dysplasia, venous, sinusoidal congestion, and kupffer cell hyperplasia in hepatic tissue.[59] However, within COE, regeneration of cells was noted; this compromised the tissue damage. These results summarize the effect of COE checking on doxorubicin-induced organ toxicity. Similarly, EAC and DOX exposed groups pointed towards tubular and glomerular congestion, glomerular atrophy, tubular cell swelling, inflammation, widening bowmen space, and cytoplasmic vacuoles; ameliorated *via* the COE treatment, whether independent or in combination with doxorubicin. Further, the observed cytotoxicity was also confirmed by histopathological devastation of the tumor mass; reduced tumor growth, decreased mitotic pattern, increased necrosis, and the occurrence of apoptotic nuclei *via* COE treatment.

Apart from *in vitro* and *silico* studies, we also confirmed the anticancer as well as organ protective activity of COE during DOX chemotherapy *via in vivo* study. These provide valuable clues in designing further clinical studies.

***In silico* studies**

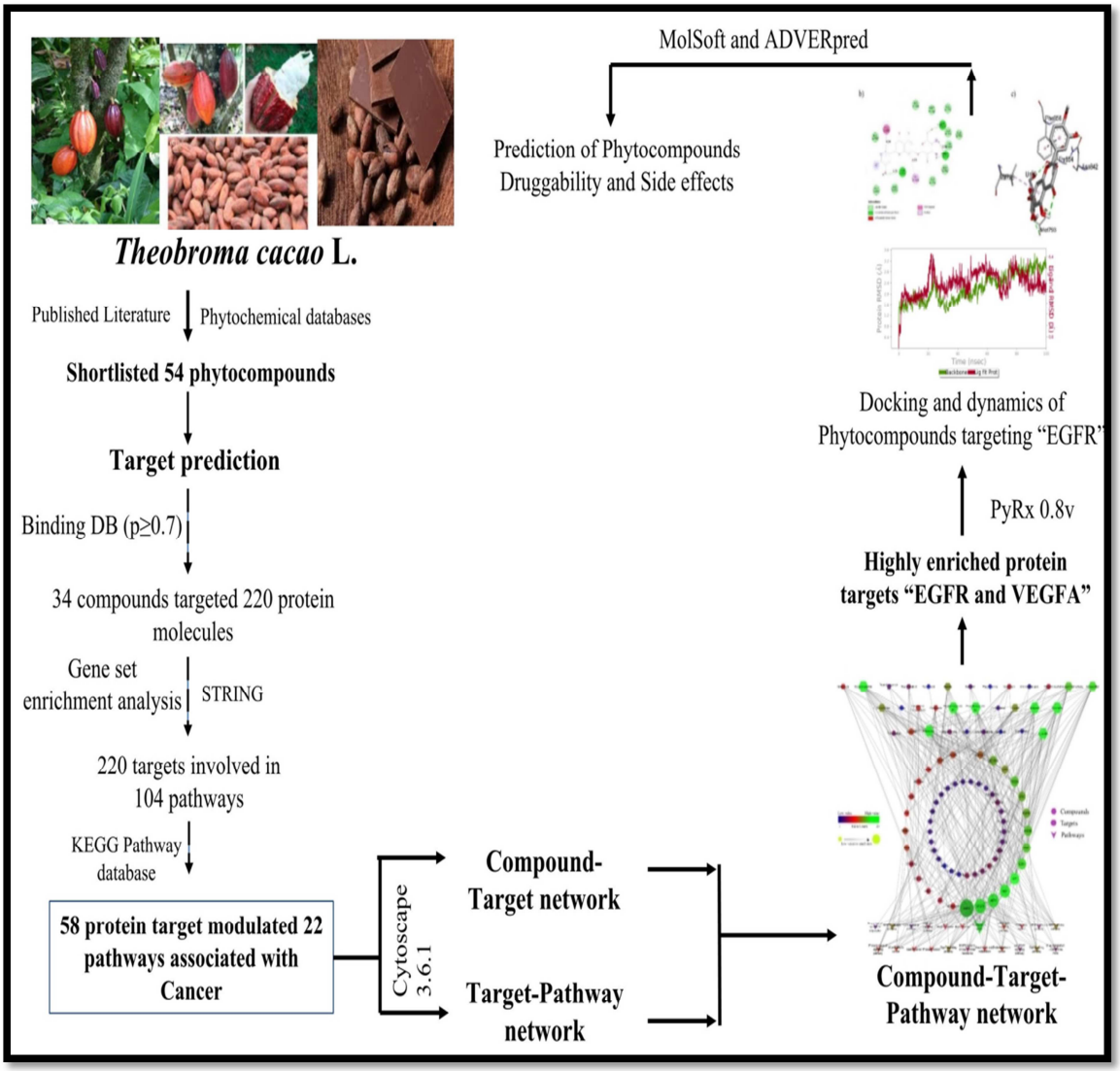


Figure 48: Graphical representation of *Insilico* studies

Ehrlich ascites carcinoma model

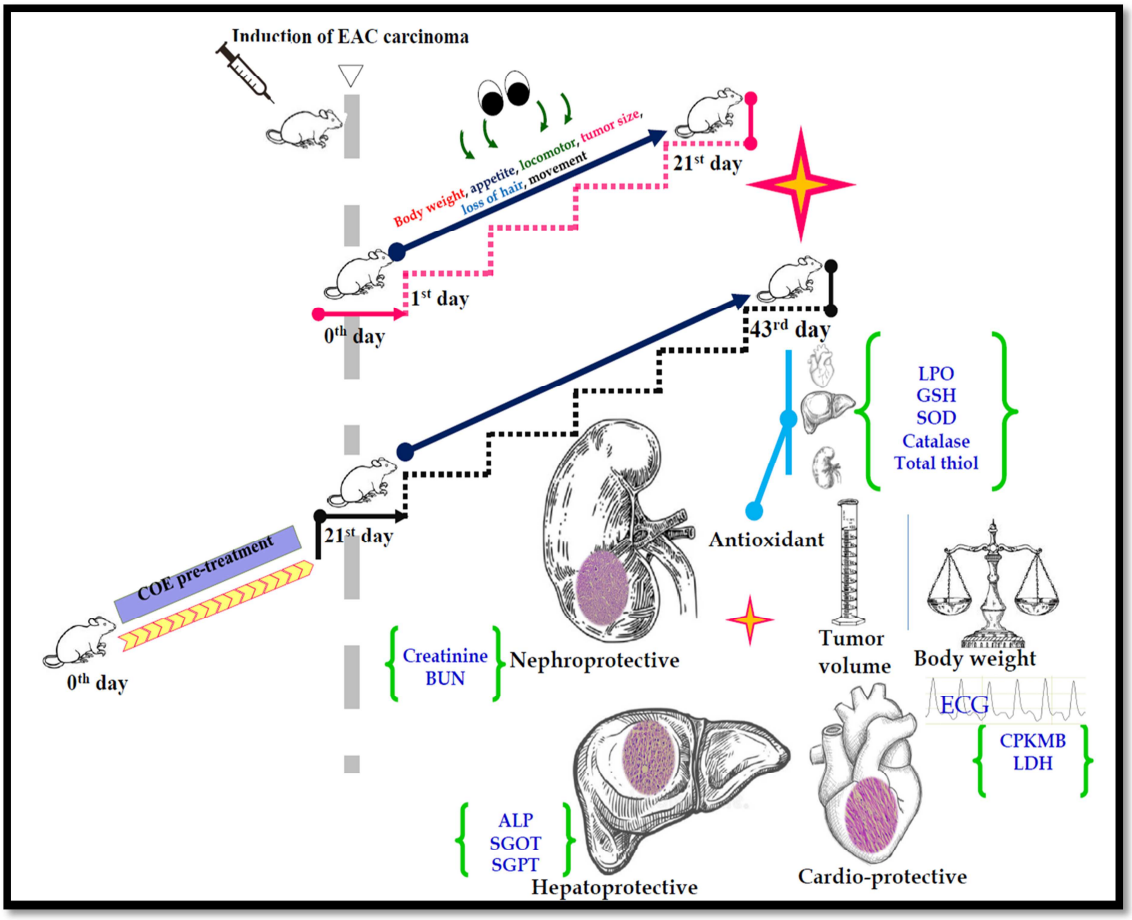


Figure 49: Graphical representation of Ehrlich ascites tumor model

Ehrlich ascites carcinoma solid tumor model

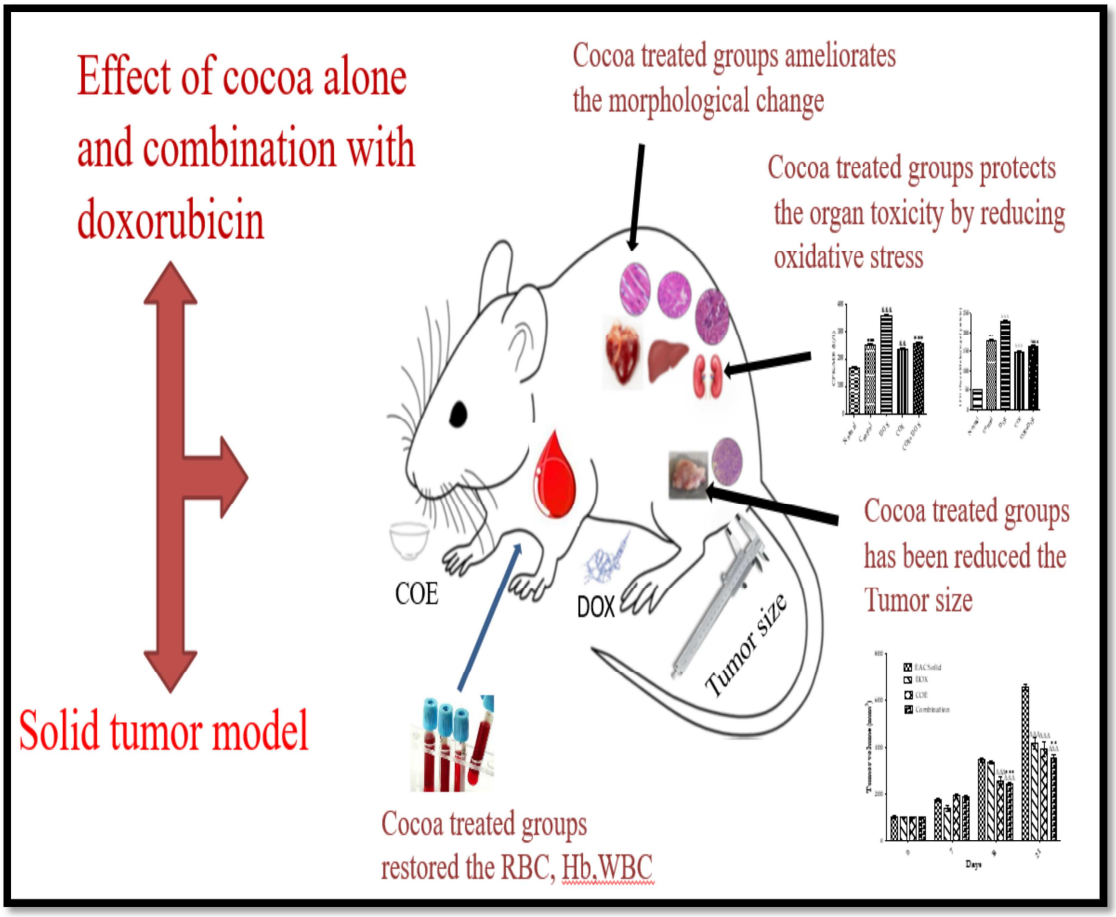


Figure 50 Graphical representation of Ehrlich ascites carcinoma solid tumor model

7. CONCLUSION

The present study not only confirmed the protective efficacy of cocoa extract against DOX-induced organ (heart, liver, and kidney) toxicities in mice but also established its anticancer activity without compromising doxorubicin efficacy in the EAC model followed by in silico approach and *in vitro* evaluation to investigate the antioxidant and anticancer activity of the cocoa. Our study demonstrated the ability of COE to neutralise the free radicals generated from DOX and maintain cellular integrity, along with its inherent anticancer properties, to increase the survival time of EAC mice. Overall, COE was found to possess promising cardioprotective, hepatoprotective, and renoprotective activities when supplemented with DOX. Furthermore, we reported interactions of the bioactive from the cocoa with a protein involved in the pathogenesis of cancer which was identified by modulation of multiple pathogenesis/ proteins involved in the ROS system and cancer pathogenesis. Gene set enrichment analysis identified the arachidonic acid pathway as a likely major target of the bioactives of cocoa to counteract cancer and oxidative stress. Hirsutrin, hyperosid, and other key constituents present in cocoa were found to target and bind with EGFR suggesting their probable roles in inhibiting EGFR and other key protein targets in cancer biology. Further, antioxidant activity validation by *in vitro* study cytotoxicity assay demonstrated higher toxicity of COE to EAC compared to CHO cell line. This means that the phytoconstituents contained in bioactives can have a stronger tendency to inhibit the growth of tumor cells than normal cells. Further, confirmatory studies at the clinical level are needed to establish cocoa as a nutraceutical against doxorubicin-induced organ damage.

8. SUMMARY

Cancer is a widespread term nowadays and a significant contributor to morbidity and early mortality. There are numerous cancer treatment options. The majority of patients receive a variety of therapies, such as surgery combined with chemotherapy and/or radiation therapy. Doxorubicin, an anthracycline derivative, is an anticancer medication used to treat several forms of cancer. The clinical use of this drug is very limited because of the peculiar and often irreversible organ toxicity (cardiotoxicity (cardiomyopathy), hepatotoxicity, and nephrotoxicity) associated with the total dose of the drug.

The mechanism of organ toxicity and the antitumor activity of doxorubicin differ, allowing organ protection without compromising its antitumor efficacy. Although there are various strategies to prevent doxorubicin-induced organ toxicity, herbal preparations are preferred because of their economy, efficacy, availability, and safety. Hence, the study's objective is to evaluate the protective activity of cocoa against Doxorubicin-induced organ toxicities (heart, liver, and kidney) and to assess the cytotoxic activity of Doxorubicin in combination with cocoa in an in mouse bearing ascites & solid tumor

Plants were procured and authenticated, followed by preliminary pharmacognostic investigations, the preparation of hydroalcoholic extracts, and phytochemical investigations of extracts were done. Further, *via in silico* approaches, we identified cocoa compounds to target protein molecules and pathways associated with cancer and oxidative stress and, majorly, EGFR was identified as the hub protein target modulated by compounds *In vitro* assay showed that cocoa selectively inhibits the proliferation and enhances the Doxorubicin anticancer activity in cancer cell lines

compared to normal cell lines. Furthermore, the pharmacological evaluation was carried out on doxorubicin-induced organ toxicity in the murine ascites model. Treatment with COE alone and in combination with DOX in tumour-bearing mice showed a significant deceleration of cancer progression and organ protection when compared to therapy with DOX alone. Also, COE alone and in combination with DOX resulted in a significant increase in the mean survival time of mice compared to treatment with DOX alone and the EAC group.

The present study not only confirmed the protective efficacy of cocoa extract against DOX-induced organ (heart, liver, and kidney) toxicities in mice but also established its anticancer activity without compromising doxorubicin efficacy in the EAC model.

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ANNEXURE - A

Authentication letter of *Theobroma cacao* L.whole plant

- **Certificate of plants authentication**

Theobroma cacao nibs were authenticated at Indian Council of Medical Research-National Institute of Traditional Medicine (ICMR-NITM) Belagavi-590010, India; and deposited the herbarium in ICMR-NITM. (Voucher number RMRC-1392)

राष्ट्रीय पारम्परिक चिकित्साविज्ञान संस्थान
ICMR-NATIONAL INSTITUTE OF TRADITIONAL MEDICINE
(भूतपूर्व क्षेत्रीय आयुर्विज्ञान अनुसंधान केन्द्र Formerly Regional Medical Research Centre)
Nehru Nagar, Belagavi-590 090

Dr. Harsha Hegde
Scientist-D
harshah@icmr.gov.in

भारतीय आयुर्विज्ञान अनुसंधान परिषद
INDIAN COUNCIL OF MEDICAL RESEARCH
स्वास्थ्य अनुसंधान विभाग, स्वास्थ्य और परिवार कल्याण मंत्रालय, भारत सरकार
Department of Health Research,
Ministry of Health & Family Welfare, Govt. of India

Date: 22-05-2018

AUTHENTICATION

This is to authenticate that the plant submitted by Ms. Priyanka Patil, Research Scholar, Dept. of Pharmacology and Toxicology, KAHER's College of Pharmacy, Belagavi is identified as ***Theobroma cacao* L.** belonging to family Malvaceae. The voucher specimen of the same has been deposited in our herbaria with accession number RMRC-1392.

Harsha Hegde
Scientist 'D'

ANNEXURE - B

Animal Ethical Approval certificate

- **Ethical approval for animal studies**

This animal study performed in the present study was performed after receiving the ethical clearance from Institutional Animal Ethics Committee (IAEC) of ICMR-NITM Belagavi; resolution number IAEC/ICMR-NTM BGM/2018/2019/3, Reg. No. 1388/GO/Re/S/ 10/ CPCSEA,Res.28-12/10/2019.

ICMR-National Institute of Traditional Medicine
(Formerly Regional Medical Research Centre)
Nehru Nagar, Belgaum - 590 010

Institutional Animal Ethics Committee
Reg. No. 1388/GO/Re/S/10/CPCSEA
Resolution No. IAEC/ICMR-NTM BGM/2018/3

Date: 21-07-2018

CERTIFICATE

This is to certify that the project proposed by Dr. Subarna Roy entitled "Pharmacological evaluation of Cocoa on efficacy and toxicity of doxorubicin in murine ascites and solid tumor" has been approved by the IAEC. The approval granted for:

Species	Gender	Number
Mice (Balb/C)	Either sex	102

The Investigator must report the outcome of the experiment to IAEC/CPCSEA. The approved number/ species/ gender of animals should not be changed without prior permission of CPCSEA/IAEC.

Dr. AHM Viswanath Swamy	**Dr.Bannappa S Unger**	**Dr.Subarna Roy**
CPCSEA Main Nominee	Member Secretary	Chairperson

ICMR- National Institute of Traditional Medicine, Belagavi

Institutional Animal Ethics Committee
Reg. No. 1388/GO/Re/S/10/CPCSEA
Resolution No. IAEC/ICMR-NITM BGM/2019/3 Date: 13.04.2019

CERTIFICATE

This is to certify that the research project proposed by Dr. Subarna Roy entitled "Pharmacological Evaluation of Cocoa on Efficacy and Toxicity of Doxorubicin in Murine Ascites and Solid Tumor" has been approved by IAEC. The approval granted for:

Species	Gender	Number of animals
Mice (Balb/C)	Female	168

The investigator must report the outcome of the experiment to IAEC/CPCSEA. The approved number/species/gender of animals should not be changed without prior permission of CPCSEA/IAEC.

Dr. A H M Viswanath Swamy	Dr.Banappa S Unger	Dr.Subarna Roy
CPCSEA Main Nominee	Member Secretary	Chairman

ANNEXURE - C

- **List of publications**

1. **Patil PP**, Khanal P, Patil VS, Charla R, Harish DR, Patil BM, Roy S. Effect of *Theobroma cacao* L. on the Efficacy and Toxicity of Doxorubicin in Mice Bearing Ehrlich Ascites Carcinoma. **Antioxidants**. 2022 Jun;11(6):1094**. Impact factor: 7.6**,
2. **Patil PP**, Patil VS, Khanal P, Darasaguppe HR, Charla R, Bhatkande A, Patil BM, Roy S. Network pharmacology and in vitro testing of Theobroma cacao extract's antioxidative activity and its effects on cancer cell survival. **Plos one**. 2022 Apr 14;17(4):e0259757. **Impact factor: 3.75**,

Note: The above mentioned impact factor of the journal are based on the Web of Science and Scopus data respectively are during the year of thesis submission (2022).

PLOS ONE

RESEARCH ARTICLE

Network pharmacology and *in vitro* testing of *Theobroma cacao* extract's antioxidative activity and its effects on cancer cell survival

Priyanka P. Patil[1,2], Vishal S. Patil[1,2☯], Pukar Khanal[1,3☯], Harish R. Darasaguppe[2]*, Rajitha Charla[2], Arati Bhatkande[2], Basanagouda M. Patil[1]*, Subarna Roy[2]*

1 KLE College of Pharmacy Belagavi, KLE Academy of Higher Education and Research (KAHER), Belagavi, Karnataka, India, **2** Indian Council of Medical Research- National Institute of Traditional Medicine, Belagavi, Karnataka, India, **3** Department of Pharmacology, NGSM Institute of Pharmaceutical Sciences (NGSMIPS), Nitte (Deemed to be University), Mangalore, India

☯ These authors contributed equally to this work.
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Citation: Patil PP, Patil VS, Khanal P, Darasaguppe HR, Charla R, Bhatkande A, et al. (2022) Network pharmacology and *in vitro* testing of *Theobroma cacao* extract's antioxidative activity and its effects on cancer cell survival. PLoS ONE 17(4): e0259757. https://doi.org/10.1371/journal.pone.0259757

Editor: Branislav T. Šiler, Institute for Biological Research, University of Belgrade, SERBIA

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Accepted: March 2, 2022

Published: April 14, 2022

Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Funding: This work was funded by an Intramural research grant of ICMR-National Institute of Traditional Medicine, Belagavi, awarded to SR and HRD.

Competing interests: The authors have declared that no competing interests exist.

Abstract

Theobroma cacao L. is a commercially important food/beverage and is used as traditional medicine worldwide against a variety of ailments. In the present study, computational biology approaches were implemented to elucidate the possible role of cocoa in cancer therapy. Bioactives of cocoa were retrieved from the PubChem database and queried for targets involved in cancer pathogenesis using BindingDB (similarity index $\geq$0.7). Later, the protein-protein interactions network was investigated using STRING and compound-protein *via* Cytoscape. In addition, intermolecular interactions were investigated *via* molecular docking. Also, the stability of the representative complex Hirsutrin-epidermal growth factor receptor (EGFR) complex was explored using molecular dynamics simulations. Crude extract metabolite profile was carried out by LC-MS. Further, anti-oxidant and cytotoxicity studies were performed in Chinese hamster ovary (normal) and Ehrlich ascites carcinoma (cancer) cell lines. Herein, the gene set enrichment and network analysis revealed 34 bioactives in cocoa targeting 50 proteins regulating 21 pathways involved in cancer and oxidative stress in humans. EGFR scored the highest edge count amongst 50 targets modulating 21 key pathways. Hence, it was selected as a promising anticancer target in this study. Structural refinement of EGFR was performed *via* all-atom molecular dynamics simulations in explicit solvent. A complex EGFR-Hirsutrin showed the least binding energy (-7.2 kcal/mol) and conserved non-bonded contacts with binding pocket residues. A stable complex formation of EGFR-Hirsutrin was observed during 100 ns MD simulation. *In vitro* studies corroborated antioxidant activity for cocoa extract and showed a significantly higher cytotoxic effect on cancer cells compared to normal cells. Our study virtually predicts anti-cancer activity for cocoa affected by hirsutrin inhibiting EGFR. Further wet-lab studies are needed to establish cocoa extract against cancer and oxidative stress.

antioxidants

MDPI

Article

Effect of *Theobroma cacao* L. on the Efficacy and Toxicity of Doxorubicin in Mice Bearing Ehrlich Ascites Carcinoma

Priyanka P. Patil [1,2], Pukar Khanal [1,3], Vishal S. Patil [1,2], Rajitha Charla [2], Darasaguppe R. Harish [2,*], Basanagouda M. Patil [1,*] and Subarna Roy [2,*]

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Abstract: Background and objective: Doxorubicin is a widely used chemotherapeutic agent that causes oxidative stress leading to cardiotoxicity, hepatotoxicity, and nephrotoxicity. In contrast, *Theobroma cacao* L. has been recorded as an anticancer agent and found to be protective against multiple chemical-induced organ injuries, including heart, liver, and kidney injuries. The present study investigated the possible role of extracts from *T. cacao* beans for organ-protective effects in doxorubicin-induced toxicity in mice bearing Ehrlich ascites carcinoma (EAC). Methodology: After survival analysis in rodents, cocoa bean extract (COE) was investigated for its efficacy against EAC-induced carcinoma and its organ-protective effect against doxorubicin-treated mice with EAC-induced carcinoma. Results: Significant reductions in EAC and doxorubicin-induced alterations were observed in mice administered the COE, either alone or in combination with doxorubicin. Furthermore, COE treatment significantly increased the mouse survival time, life span percentage, and antioxidant defense system. It also significantly improved cardiac, hepatic, and renal function biomarkers and markers for oxidative stress, and it also reduced doxorubicin-induced histopathological changes. Conclusion: COE acted against doxorubicin-induced organ toxicity; potent antioxidant and anticancer activities were also reflected by the COE itself. The COE may therefore serve as an adjuvant nutraceutical in cancer chemotherapy.

Keywords: anticancer; cardiotoxicity; cocoa; doxorubicin; Ehrlich ascites carcinoma; *Theobroma cacao* L.

Citation: Patil, P.P.; Khanal, P.; Patil, V.S.; Charla, R.; Harish, D.R.; Patil, B.M.; Roy, S. Effect of *Theobroma cacao* L. on the Efficacy and Toxicity of Doxorubicin in Mice Bearing Ehrlich Ascites Carcinoma. *Antioxidants* **2022**, *11*, 1094. https://doi.org/10.3390/antiox11061094

Academic Editors: Elisabetta Bigagli, Cristina Luceri and Stanley Omaye

Received: 15 March 2022
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Published: 31 May 2022

1. Introduction

Doxorubicin, one of the well-established chemotherapeutic agents, is recommended in the management of breast, liver, kidney ovarian, thyroid cancer, Wilms' tumor, and acute lymphoblastic and myeloblastic leukemia [1–6]. Doxorubicin exhibits its therapeutic activity by inhibiting the enzyme topoisomerase II and cleaving the DNA within tumor cells [7]. Despite its efficacy in managing the above-mentioned tumors and cancers, doxorubicin pharmacotherapy is limited due to its toxicity, mainly targeting vital organs, viz., the heart, liver, and kidney [8–10]. The suspected toxicity could be the outcome of the reactive oxygen species (ROS) generation and other Fenton reactions within the cytoplasm through the doxorubicin metabolite doxorubicinol [11–14].

Medicinal plants are being utilized by traditional healers to manage multiple communicable and non-communicable diseases due to the presence of various secondary bioactives, i.e., flavonoids, terpenes, alkaloids, and polyphenols [15,16]. Furthermore, due to the H-donating capacities of bioactives, extracts of various medicinal plants neutralize free radicals and terminate Fenton reactions, reducing oxidative stress in the cell [17].

ANNEXURE - D

- **List of presentations**

1. Securied **first prize**, **PRIYANKA PATIL** "*in-vitro* testing of *Theobroma cacao* extract antioxidative activity & its effects on cancer cell survival" in the national conference themed on "emerging approaches in drug discovery: artificial intelligence & drug repurposing perspective" organized by KLE college of pharmacy, Vidyanagar, hubballi, on 14th March 2022.
2. **PRIYANKA PATIL** "Structural informatics to divulge anticancer mechanisms of Theobroma cacao" in 9th international congress of society for ethnopharmacology, India(SFEC-2022) held at JSS college of pharmacy, JSS academy of higher education and research ,Mysuru, Karnataka,India from 22nd to 24th ,April2022.
3. **PRIYANKA PATIL** "Cocoa extract attenuates doxorubicin-induced cardiotoxicity in Wistar rats: a potential nutraceutical candidate for supplementation in patients undergoing doxorubicin therapy " held on 26th to 27th October 2018 at KLE college of pharmacy, Belagavi, Karnataka.

(*Certificates are attached*)

Cycle 3

B. Pharm Program

Rank Band 76-100

Category 'A'

KLE College of Pharmacy

Vidyanagar, Hubballi - 580031.

A Constituent unit of KLE Academy of Higher Education & Research, Belagavi (Deemed-to-be-University)

Certificate of Excellence

This certificate is awarded to Ms Priyanka Patil, Research Scholar, KLE College of Pharmacy / ICMR-NITM, Belagavi, for securing FIRST PRIZE, for Poster Presentation entitled "Invitro testing of *Theobroma cacao* extract antioxidative activity & its effects on cancer cell survival" in the National Conference themed on **"Emerging Approaches in Drug Discovery: Artificial Intelligence & Drug Repurposing Perspective"** organized by KLE College of Pharmacy, Vidyanagar, Hubballi, on 14th March 2022.

Dr. AHM V Swamy
Principal
KLE CoP, Hubballi

Dr. Pramod C Gadad
Convener
KLE CoP, Hubballi

Mr. Santosh B Patil
Coordinator
KLE CoP, Hubballi

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Certificate

This is to certify that

Prof./Dr./Mr./Ms. Priyanka P. Patil

has participated as ~~Resource Person~~ / Poster Presenter / Delegate and attended the scientific sessions of

National Conference on **"Advances in Drug Discovery and Development"** held on the Occasion of **Golden Jubilee Year**

Celebrations, on **26th & 27th October 2018** at KLE College of Pharmacy, Belagavi, Karnataka

Dr. B. M. Patil
Chairman - LOC

Dr. S. S. Jalalpure
Convener

JSS ACADEMY OF HIGHER EDUCATION & RESEARCH (DEEMED TO BE UNIVERSITY) MYSURU

International Society for Ethnopharmacology

Azadi Ka Amrit Mahotsav

9th SFEC 2022

22nd to 24th April 2022

International Congress of SOCIETY FOR ETHNOPHARMACOLOGY, INDIA

(Globalizing Local Knowledge and Localizing Global Technologies)

Theme : Redefining Ethnopharmacology for the Global Health and Wellbeing

Certificate of Participation

This is to certify that Prof./Dr./Mr./Ms. Priyanka Patil has Presented a PAPER in ORAL/POSTER SESSION in the 9th International Congress of the Society for Ethnopharmacology, India (SFEC-2022) held at JSS College of Pharmacy, JSS Academy of Higher Education & Research, Mysuru, Karnataka, India from 22nd to 24th, April 2022.

Title of presentation: Structural informatics to divulge anticancer mechanisms of Theobroma cacao:

Dr. T.M. Pramod Kumar	Dr. K. Mruthunjaya	Mr. Birendra K. Sarkar
Organizing Chairman, SFEC - 2022	Organizing Secretary, SFEC - 2022	President - SFE - India

NPTEL Online Certification

(Funded by the Ministry of HRD, Govt. of India)

This certificate is awarded to

PRIYANKA PATIL

for successfully completing the course

Health Research Fundamentals

(NIE-ICMR e-Certificate course: NIeCer 101)

with a score of **69 %**

Online Assignments	21.25/25	Proctored Exam	48/75

Total number of candidates certified: **580**

Dr. Manoj Murhekar
Scientist 'G' & Director
ICMR-National Institute of Epidemiology, Chennai

Prof. Balram Bhargava
Secretary to Govt. of India, Dept. of Health Research &
Director-General, Indian Council of Medical Research

Prof. Andrew Thangaraj
NPTEL Coordinator
IIT Madras

ICMR - National Institute of Epidemiology

Feb to Mar 2018
(8 week course)

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