

**MODULATORY EFFECTS OF GREEN TEA AND GREEN TEA
COMBINATION AGAINST BENZO(A)PYRENE-MEDIATED LUNG
TUMORIGENESIS IN MICE MODEL**

A Thesis

Submitted in partial fulfillment of the requirements for the
award of the degree of

DOCTOR OF PHILOSOPHY (Ph.D)

**in
(ZOOLOGY)**

By

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DECLARATION

I hereby declare that this thesis entitled “**Modulatory Effects of Green tea and Green tea Combination Against Benzo(a)pyrene-mediated Lung Tumorigenesis in Mice Model**” is an original report of my research, has been written by me and has not been submitted for any previous degree. The experimental work has been carried out at school of Bioengineering & Biosciences, Lovely Professional University, Phagwara, Punjab, India under the guidance of **Dr. Amit Sehgal (16824)**, Assistant Professor, School of Bioengineering and Biosciences, Lovely Professional University, Phagwara, Punjab, India, for the award of the degree of doctor of philosophy (PhD) in Zoology.

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CERTIFICATE

This is to certify that the thesis entitled “**Modulatory Effects of Green tea and Green tea Combination Against Benzo(a)pyrene-mediated Lung Tumorigenesis in Mice Model**” that is being submitted by **Sumaya Farooq** in partial fulfillment of the requirements for the award of degree “**DOCTOR OF PHILOSOPHY (Ph.D)**” in “**ZOOLOGY**”, is a record of bonafide and authentic work done under my supervision. The contents of this research have neither been taken from any other source nor have been submitted to any other Institute or University for award of any degree or diploma. This thesis is fit for submission and the partial fulfillment of the conditions for the award of degree of PhD in zoology.

Signature of Supervisor

Dr. Amit Sehgal,

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*This Thesis is dedicated to My
Dear Parents & My Sisters*

ABSTRACT

Green tea a commonly consumed beverage throughout the world has gained popularity due to its various health promoting properties. The meta-analysis reports proposed that green tea (GT) consumption is related with mild to moderate effects on four major global killers such as cancer, type II diabetes, stroke, and atherosclerosis-related cardiovascular events. The green tea health promoting power can be increased by combining it with other medicinal plants used as herbal teas or tisanes. The current study was undertaken to determine the antioxidant, antiangiogenic and lung cancer preventive activities of green tea alone and in combination with herbal teas [*Ocimum gratissimum* (OG), *Cymbopogon citratus* (CC), *Cymbopogon flexuosus* (CF), and *Hibiscus rosa-sinensis* (HR)]. The antioxidant activities of aqueous infusions were determined using cell free assays (DPPH, ABTS and NO) and *ex-vivo* models (lipid peroxidation test and haemolysis assay). The antioxidant interactions of green tea and herbal tea were analyzed based on combination index values (CI), isobolograms and polygonograms. The total phenolic and flavonoid content was measured by employing Folin-Ciocalteu reagent and aluminium chloride colorimetry assay. The ATR-FTIR and UV-VIS analysis of all infusions alone or as binary mixtures was carried out. The inhibition of new blood vessel formation (angiogenesis) was determined by chick chorioallantoic membrane assay. The green tea and herbal tea pair illustrated highest antioxidant and antiangiogenic ability was further scrutinized for lung cancer chemoprevention *in vitro* by determining the cytotoxicity against A549 lung cancer cell line, and *in vivo* determination of benzo(a)pyrene [B(a)P] induced pulmonary lesions in mice.

The results revealed that GT and OG combination (GT+OG) showed highest antioxidant potential as compared to other infusions. The antioxidant interaction ranged from synergistic to additive between GT and herbal teas. GT and OG combination exhibited maximum synergistic interaction in all the performed antioxidant assays. A significant difference was noticed in the blood vessel formation between the VEGF (vascular endothelial growth factor) treated, and both VEGF and infusion treated groups. GT and GT+OG were found to be most effective in inhibiting VEGF induced angiogenesis. The TPC was found maximum in GT followed by OG, HR, GT+CF, GT+OG, GT+CC, GT+HR, CF and minimum in CC. Whereas, TFC was observed to be high in GT, GT+CF, OG followed by GT+HR, GT+OG, GT+CC, HR, CF and

lowest in CC. The ATR-FTIR spectra in the mid infrared region ($4000-600\text{cm}^{-1}$) demonstrated multiple peaks with varying intensity for various infusions. The maximum peak intensity was noticed between $3000-3600\text{ cm}^{-1}$ for GT and GT combinations except few additional minor peaks in GT combinations in the region $3600-4000\text{ cm}^{-1}$. The UV-VIS absorbance spectra of green tea showed an intense peak in the region (250-300 nm) while for other infusions lower intensity peaks were observed. GT combinations manifested an intense peak in the region of 250-300nm, and GT+OG demonstrated the highest absorption peak followed by GT+HR, GT+CF and GT+CC.

The binary combination of GT and OG was selected for further evaluation at five different ratios (3:1, 2:1, 1: 1, 1:2 and 1:3) for their antioxidant potential. The equal proportion of GT+OG (1:1) exhibited higher radical scavenging activity, TPC and TFC. GT and OG mixture demonstrated interactions ranged from moderate antagonism to strong synergism at various ratios with maximum synergism displayed by 1:1. The different ratios revealed similar FTIR spectra with minor difference in broadness and intensities of peaks, and a slight shifting in the wavenumbers. The UV- VIS spectra showed the highest absorption peak in the region (250-300 nm) for GT followed by 3:1, 1:1, 2:1, 1:2, 1:3 and the least absorbance was noticed for OG.

The GT, OG and GT+OG (1:1) were further assessed for lung cancer chemoprevention. The aqueous infusions of GT and GT+OG displayed higher cytotoxic ability against A549 lung cancer cell line as compared to OG at low concentration whereas no significant difference was detected at higher doses. The *in vivo* results revealed that administration of GT, OG and GT+OG augmented plasma antioxidant status in carcinogen [B(a)P] injected animals. GT+OG showed higher plasma radical scavenging potential than GT and OG. The histopathological examination exhibited that all the three infusions (GT, OG and GT+OG) demonstrated similar protective effect against B(a)P induced hyperplastic zones. Thus, this study provides a scientific basis for designing and formulating beverages containing GT combinations based on their antioxidant interactions that can potentially enhance the efficacy of GT to reduce oxidative stress.

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CHAPTER 1
INTRODUCTION

INTRODUCTION

Cancer is the world's most critical cause of illness and deaths (Torre et al., 2016; Siegel et al., 2019). It is a group of ailments in which cells have the capacity to grow continuously and spread to other body parts (Kohler et al., 2015). In India, the total number of deaths reported due to cancer is 7.8 lakh in 2018 (Ferlay et al., 2019). Among the different types of cancers, lung cancer is the major killer throughout the world, and ranked at fourth position for cancer related deaths in India (Globocan, 2018). Lung cancer was rare in the early 20th century, but its prevalence and mortality rate increased due to the increased trend in smoking (Centers for Disease Control, 2005). Most of the deaths associated with lung cancer occur because of late diagnosis, poor prognosis and less effective treatments (Uramoto and Tanaka, 2014). The etiology of lung cancer includes various factors like long term exposure to tobacco smoke, disclosure of radiations, chloromethyl ethers, heavy metals and polycyclic aromatic hydrocarbons (PAHs) (Zhao et al., 2006; Thun et al., 2008; Wild et al., 2009). Tobacco smoking is one of key risk factors for lung cancer (Yanbaeva et al., 2007). Tobacco smoke contains over 7,000 chemical substances among which 69 can cause cancer to laboratory animals and humans (Hecht, 2006). These chemicals are categorized as heterocyclic compounds, PAHs, heterocyclic aromatic amines, aromatic amines, nitrosoamines, phenolic compounds, volatile hydrocarbons, and nitrohydrocarbons, miscellaneous inorganic and organic compounds (IARC, 1973, 1983).

Polycyclic aromatic hydrocarbons (PAHs) are a category of organic contaminants with mutagenic, carcinogenic and teratogenic properties and are unsafe to humans (Xu et al., 2011). PAHs exposure causes cancer in different organs of humans, such as skin, lung, bladder and colon (IARC, 2010). The major sources of PAHs are cigarette smoke, grilled/barbecued, smoke cured meats, smokeless tobacco and incomplete combustion of organic matter (IARC, 2010). Benzo(a)pyrene [B(a)] is one of the most commonly researched PAHs (Besaratina and Pfeifer, 2003). It was reported to be the first carcinogen investigated in cigarette smoke. It was categorized by International Agency for Research on Cancer (IARC) as group 1 carcinogen (enough data to support B(a)P being carcinogenic to humans) (IARC, 1983, 2010). It is a pro carcinogen that after metabolic activation through cytochrome P450 enzymes gets transformed into vital carcinogen, B(a)P-7,8-diol-9,10-epoxides (BPDE). BPDE interacts with the DNA and

creates covalent adducts (Gelboin, 1980; Kasala et al., 2015). If these DNA adducts remain unrepaired, it may lead to mutations responsible for cancer (Pfeifer et al., 2002). B(a)P chemical processing in the body generates excessive reactive species that are implicated with oxidative DNA damage (Penning et al., 1999; Kasala et al., 2015). Oral or intraperitoneal administration (single or multiple) of B(a)P in animal models demonstrated formation of pulmonary lesions or tumors (Kasala et al., 2016).

Although considerable advances are made in the field of lung cancer therapy (chemotherapy, radiotherapy, targeted therapy and immunotherapy) but the average five year survival rate is lowest in lung cancer (31%) amongst the five most common cancers i.e. breast (75%), bladder (74%), prostate (69%) and colorectal (51%) (Niyazi et al., 2011; Iwamoto, 2013). The burden of lung cancer is projected to be a major problem worldwide in the coming years, especially in East Asia due to the rising population (Bray et al., 2018). There is an immediate need to establish effective approaches for cancer prevention and treatment, with less or no toxic effects (Shu et al., 2013; Govind and Madhuri, 2006). The use of natural agents capable of preventing, stopping or reversing carcinogenesis can paved the way to tackle this dreadful disease (Yan et al., 2006). Carcinogenesis involves molecular and histological alterations in the effected organ (Herzig and Christofori, 2002). There is instability in genetic makeup, modulation in expression of oncogenes, tumor suppressors genes and DNA repair genes (Feo, 2014). Previous studies illustrated that the risk of cancer can be influenced by dietary factors (Soldati et al., 2018; Davis et al., 2019). Numerous population based studies revealed, people who eat five portions of fruits and vegetables a day are at around half the risk of developing cancers, particularly digestive and respiratory cancers (Surh, 2003). Vegetables and fruits are rich in phytochemicals that can inhibit carcinogenesis through various mechanisms such as enhancing the chemical detoxification, modifying the absorption and metabolism of carcinogen, quenching the reactive oxygen species (ROS), declining DNA destruction, decreasing angiogenesis, modulation of gene expression and apoptotic induction **Fig. 1** (Garg et al., 2008; Rajendran et al., 2008). From various earlier studies it was found that tea consumption is related with various health benefits, such as chemoprevention of cancers, heart and liver diseases (Chen and Dou, 2008; Yang et al., 2009).

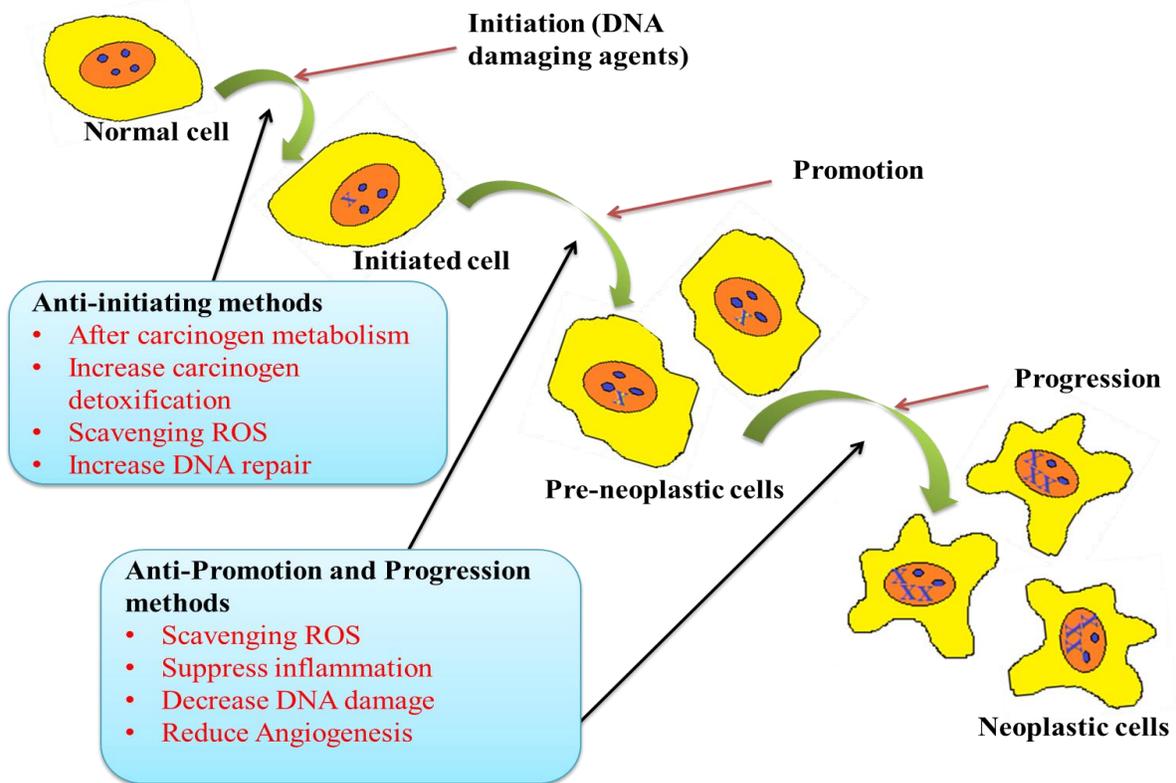


Fig. 1: Major steps involved in carcinogenesis and chemoprevention (Hursting et al., 1999).

After water, tea made from the *Camellia sinensis* leaves is the most commonly consumed drink (Karak et al., 2017). Green tea, the minimally processed form of tea is attaining popularity all around the world (Senanayake, 2013). It is obtained from the steamed or panfried leaves of the Theaceae family of *Camellia sinensis* plants (Schneider and Segre, 2009). It is known to possess protective effects against various diseases like obesity, arteriosclerosis, diabetes, cancer, allergy, neurodegenerative diseases, hepatitis, viral and bacterial infection (Kakuda, 2002; Suzuki et al., 2000; Wolfram et al., 2006; Friedman, 2007; Ciesek et al., 2011; Tang et al., 2013; Khan and Mukhtar, 2013). But meta-analysis studies revealed the fact that green tea consumption is linked with limited effects on four major worldwide killers (Zhong et al., 2001; Tang et al., 2009; Johnson et al., 2012; Wang et al., 2014). Green tea's potential for health promotion can be enhanced by supplementing it with other medicinal plants commonly used as herbal tea. The increasing awareness of health about the role of diet in an individual general well-being was the key reason of selection for most of the herbal teas (Joubert et al., 2017). Herbal teas or tisanes are beverages that are made of different parts of plants other than *C.*

sinensis. They have been used to prevent and cure diseases for thousands of years (Deetae et al., 2012). They are easy, reliable, low-cost, caffeine - and drug - free ways to get taste and different health benefits from herbs (Killedar and Pawae, 2017). Oral administration of more than one herb or phytochemical can influence the initiation, promotion and progression of cancer development by affecting and targeting the complementary mechanisms (Gawlik- Dziki, 2012). Combinations of green tea, green tea polyphenols, and green tea with drugs or other herbs can inhibit carcinogenesis when given during initiation, post initiation or during entire experiment (Suganum et al., 2011; Lambert, 2013).

The combination of various plant-based foods can exhibit additive, synergistic, or antagonistic interactions, which in turn can affect their biological activities (Gawlik-Dziki, 2012). The antioxidant synergistic interactions between teas and herbal infusions will increase the potential for disease prevention correlated with redox imbalance (Phan et al., 2018). Combined effect of green tea with its polyphenols or other herbs proved to be more beneficial in cancer chemoprevention than the treatment with individual compound (Eckert et al., 2006; Khan and Mukhtar, 2010; Gao et al., 2013).

In current study, the binary mixtures of green tea with *Ocimum gratissimum* (Vana Tulsi), *Cymbopogon citratus* (West Indian lemongrass), *Cymbopogon flexuosus* (East Indian lemongrass) and *Hibiscus rosa – sinensis* (China rose) are evaluated for their possible synergistic antioxidant interaction, bearing in mind how these aqueous infusions are generally prepared before consumption. The green tea combination demonstrating highest antioxidant activity was further tested for cancer preventive potential employing benzo(a)pyrene mediated lung carcinogenesis in murine model.

CHAPTER 2

REVIEW OF LITERATURE

REVIEW OF LITERATURE

2.1 Cancer

Cancer is world's one of the highest growing health problems, and is the second most common reason of mortality in developing countries (Siegel et al., 2019). Every sixth death worldwide is caused because of cancer that makes it the second prevalent cause of death next to cardiovascular diseases only (Schutte, 2017). The estimated figure of new cases of cancer have risen to 18.1 million with a death toll of about 9.6 million in 2018 (Globocan, 2018; Sedighi et al., 2019). According to IARC 2018, the global cancer load is expected to increase by 2040 to 27.5 million (new cases) of cancer and 16.3 million (deaths), due to increased population progression and the aging. In India, 7.8 lakh deaths are estimated due to cancer which includes 4.1 lakh deaths in men and 3.7 lakh in women (Ferlay et al., 2018). Throughout the world, a total of 23.4% cancer cases and 20.3% cancer deaths were reported in Europe as it owns only 9.0% of the worldwide population. However, in developed countries such as America accounts about 21.0% of the incidence rate and 14.4% of deaths worldwide as it has 13.3% of world population. In comparison to other regions (Americas, Africa and Oceania) of the world, the proportion of deaths (57.3%) in Asia and (7.3%) in Africa are greater than the proportion of incident cases such as (48.4%) and (5.8%), respectively. The reasons for this disparity are larger prevalence of certain types of cancer that are associated with poorer prognosis and advanced mortality rates, imperfect diagnostic and treatment facilities in many countries as depicted in **Fig. 2** (Globocan, 2018). Globally, the prevalence and death rate of cancer is highly increasing that could be due to numerous factors such as aging, dietary supplements, growth of the population and carcinogens (Danaei et al., 2005; Gersten and Wilmoth, 2002; Libby and Kobold, 2019). Despite the accessibility of innovative advances in pharmaceutical therapeutics in the world there is still high mortality rate caused by cancer each year (Sedighi et al., 2019). Cancer is a multi-step process that can be initiated by various internal and external elements such as hereditary mutations, hormones and immune disorders, environmental pollutants, tobacco and infectious agents (Siegel et al., 2016; Ferlay et al., 2018).

Cancer is linked to high disease and death rates globally, so there is a vital need to discover the methods of tackling such diseases where the recent approaches of treatment are mainly radiation-based therapy, gene therapy/or hormonal therapy, chemotherapy and surgery

(Rady et al., 2017). In 2018, the 5 years prevalence is estimated to be 43.8 million, as cancer prevalence is the number of people alive who had a diagnosis of cancer during a specified period of time (Globocan, 2018).

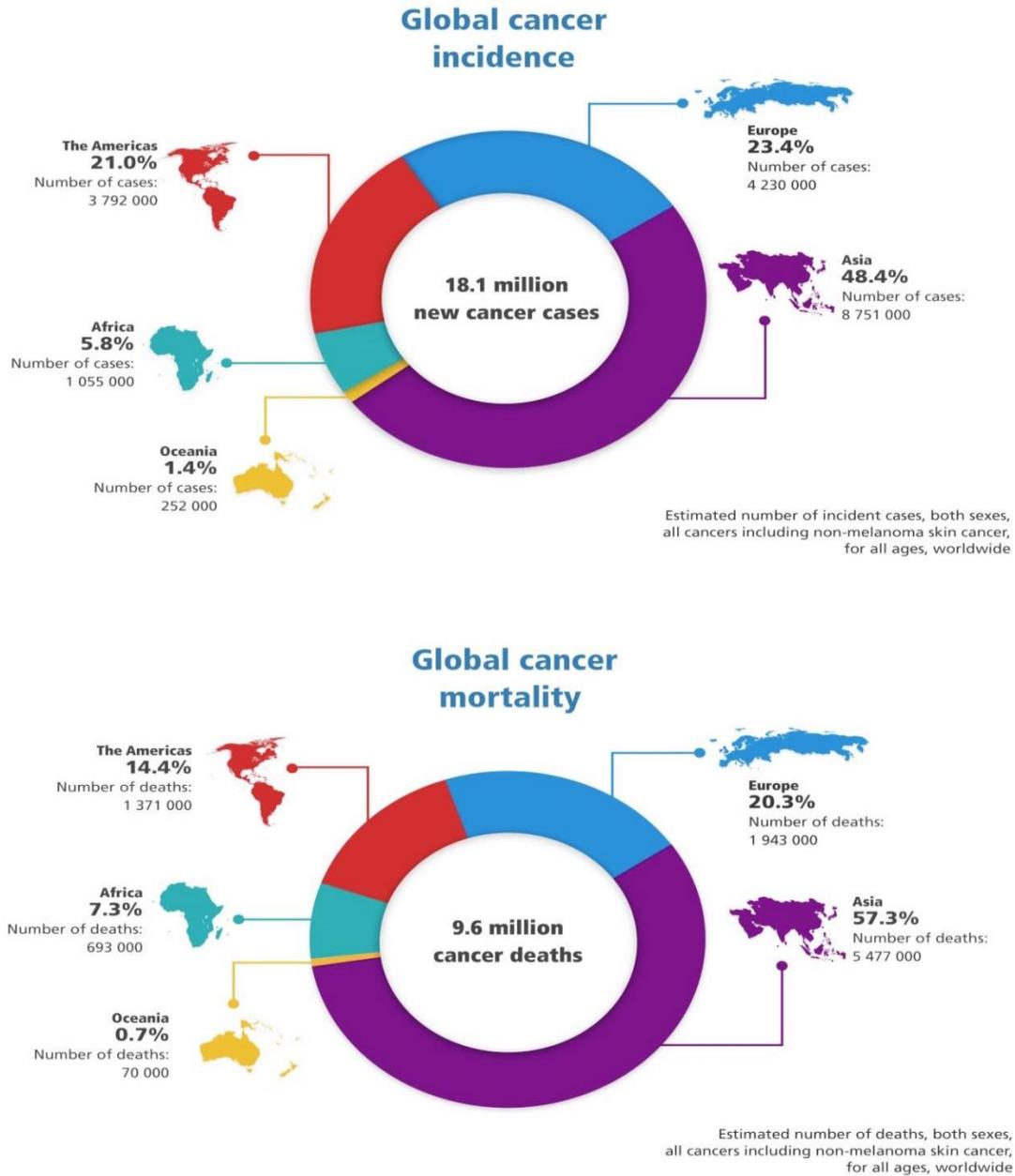


Fig. 2: Estimated global cancer occurrence and death rate in 2018 (Globocan, 2018).

2.2 Lung Cancer

Among all type of cancers, globally the lung cancer is the leading cause of deaths from cancer (Greenlee et al., 2001). Lung cancers have found to take place at high rate in both well developed and less developed countries (Parkin et al., 2002). The estimated number of cancer cases were highest for lung cancer and breast cancer (11.6% and 11.6%) followed by colorectum cancer (10.2%), prostate cancer (7.1%), stomach cancer (5.7%), liver cancer (4.7%), esophagus cancer (3.2%), cervix uteri cancer (3.2%) and other cancers (42.9%), respectively for 2018 **Fig. 3**. However, the most common cause of cancer deaths by a single type of cancer is lung cancer accounts (18.4%), that is followed by colorectum (9.2%), stomach (8.2%), liver (8.2%), breast (6.6%), oesophagus (5.3%), pancreas (4.5%), prostate (3.8%) and other cancers (35.8%), respectively for 2018 (Globocan, 2018) as illustrated in **Fig. 4**.

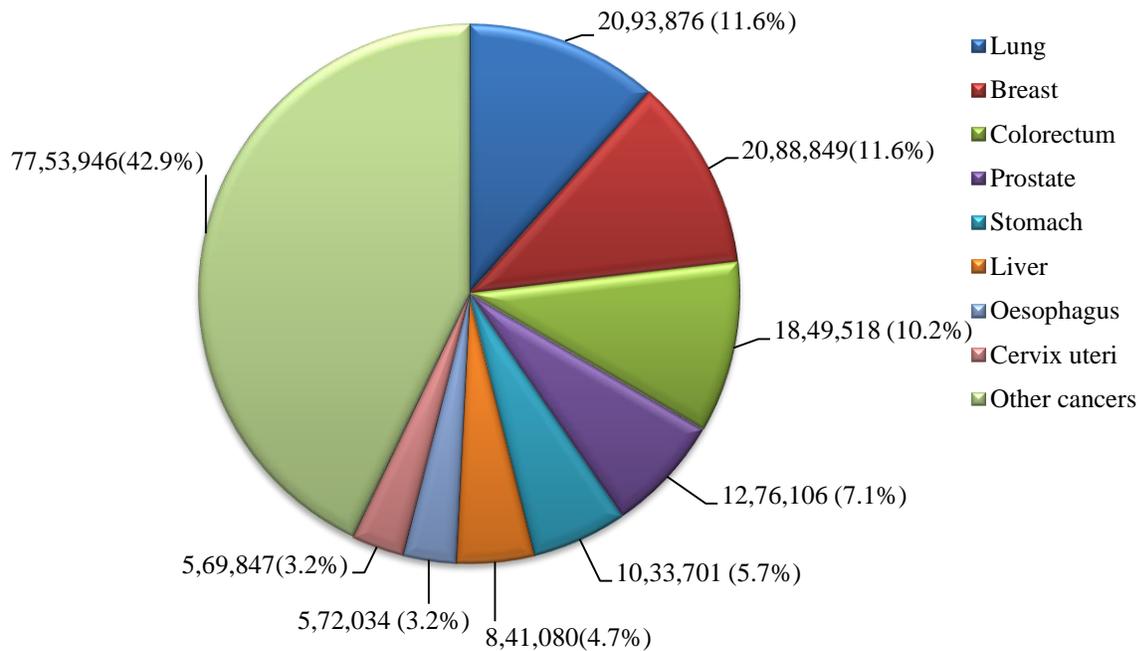


Fig. 3: Estimated new cancer cases in both males and females of all ages for 2018 (GLOBOCAN, 2018).

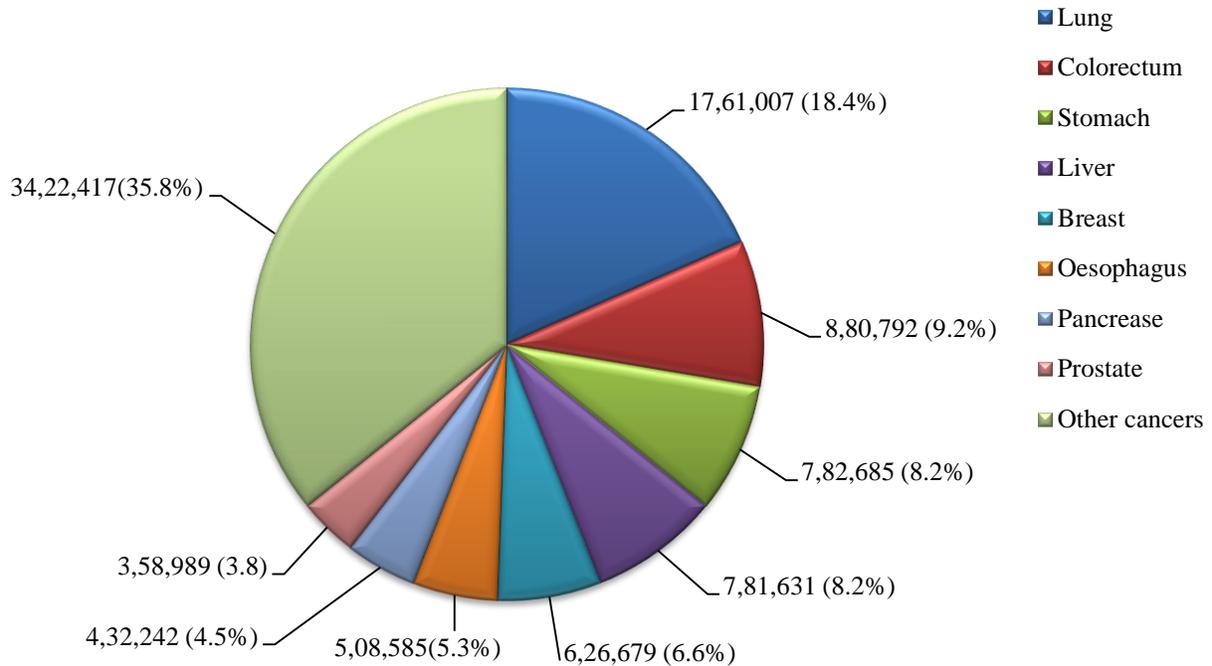


Fig. 4: Estimated cancer deaths in both males and females of all ages for 2018 (GLOBOCAN, 2018)

There are about 18.1 million new cases (9.5 million in men and 8.6 million in women) and 9.6 million deaths (5.4 million in men and 4.2 million in women) were reported for ten most common cancers. In men the incidence and mortality rate of lung cancer was found to be higher and rest are given in the decreasing order: (prostate and liver cancer) > (colorectum and stomach cancer) > (stomach and colorectum cancer) > (liver and prostate cancer) > (bladder and esophagus cancer) > (esophagus and pancreas cancer) > (non- hodgkin lymphoma and leukaemia) > (kidney and bladder cancer) and (leukaemia and non- hodgkin lymphoma), respectively. While as in females both incidence and death rate was higher in breast cancer and other are given as: (colorectum and lung cancer) > (lung and colorectum cancer) > (cervix uteri cancer) > (thyroid and stomach cancer) > (corpus uteri and liver cancer) > (stomach and pancreas cancer) > (ovary cancer) > (liver and esophagus cancer) and (non- hodgkin lymphoma and leukaemia), respectively as shown in **Table 1**. In a survey of 185 countries, the lung cancer was found to be the leading cause of mortality (93 countries) shadowed by prostate and liver cancer (46 and 20 countries) in men. However, the third most prominent cause of cancer deaths

(28 countries) is the lung cancer in women, cervical cancer (42 countries) is the second one and breast cancer (103 countries) is the first foremost cause of cancer mortality (Bray et al., 2018). Lung cancer type diagnosis depends on the type of histoarchitecture of tumor and the presence or absence of metastatic deposits and are usually detected at the late stages where survival rate of the patient is very low (Folch et al., 2015). A five-year survival rate is measure of how many people stay alive with lung cancer after five years of diagnosis (Howlader et al., 2019). The lung cancer survival rate is influenced by many factors such as age, general health, gender, race and treatment used (Tas et al., 2013; Pinto et al., 2018). GLOBOCAN 2018, stated the number of lung cancer incidence, mortality and the five year prevalence of both sexes in Asia, North America, Latin America, Europe and Africa, Caribbean and Oceania. Asia the largest continent of the world demonstrated higher number of lung cancer incidences (58.5%), death rate (60.7%) and 5 year of prevalence (56.6%) followed by Europe (22.4%, 22% and 23.3%), North America (12.1%, 9.8% and 13.3%), Latin America and Caribbean (4.3%, 4.6% and 4.0%), Africa (1.9%, 2.1% and 1.8%) and Oceania (0.81%, 0.67% and 0.87%), respectively as depicted in **Table 2**

Table 1: Estimated incidence and death cases for the 10 most common cancers in 2018 for males and females (GLOBOCAN, 2018)

| Type of cancer | Males | | Females | |
|----------------|---------------|---------------|---------------|---------------|
| | Incidence (%) | Mortality (%) | Incidence (%) | Mortality (%) |
| Lung | 14.5 | 22.0 | 8.4 | 13.8 |
| Breast | - | - | 24.2 | 15.0 |
| Colorectum | 10.9 | 9.0 | 9.5 | 9.5 |
| Prostate | 13.5 | 6.7 | - | - |
| Stomach | 7.2 | 9.5 | 4.1 | 6.5 |
| Liver | 6.3 | 10.2 | 2.8 | 5.6 |
| Esophagus | 4.2 | 6.6 | - | 3.6 |
| Thyroid | - | - | 5.1 | - |

| | | | | |
|------------------------------|------|------|------|------|
| Bladder | 4.5 | 2.8 | - | - |
| Leukaemia | 2.6 | 3.3 | - | 3.1 |
| Kidney | 2.7 | - | - | - |
| Non- Hodgkin lymphoma | 3.0 | 2.7 | 2.6 | - |
| Ovary | - | - | 3.4 | 4.4 |
| Corpus uteri | - | - | 4.4 | - |
| Cervix uteri | - | - | 6.6 | 7.5 |
| Pancreas | - | 4.2 | - | 4.9 |
| Others | 30.6 | 23.0 | 28.9 | 23.1 |

Source: GLOBOCAN, 2018

Incidence: is the number of new cases occurring in a given time and geographical area, either as the overall number of cases per year or as the average per 100,000 individuals per year.

Mortality: the number of deaths occurring in a given region and period, and the death rate is the number of deaths per 100,000 individuals per year.

Table 2: Estimated number of lung cancer occurrence, mortality and 5-year prevalence of both sexes.

| Population | Incidence (Both sexes) | Mortality(Both sexes) | 5-year prevalence (both sexes) |
|------------------------------------|-------------------------------|------------------------------|---------------------------------------|
| Asia | 1225029 (58.5%) | 1068862 (60.7%) | 1206528 (56.6%) |
| Europe | 470039 (22.4%) | 387913 (22%) | 497283 (23.3%) |
| North America | 252746 (12.1%) | 173278 (9.8%) | 283985 (13.3%) |
| Latin America and Caribbean | 89772 (4.3%) | 81384 (4.6%) | 85456 (4.0%) |
| Africa | 39353 (1.9%) | 37748 (2.1%) | 38144 (1.8%) |
| Oceania | 16937 (0.81%) | 11822 (0.67%) | 18568 (0.87%) |
| Total | 2093876 (100%) | 1761007 (100%) | 2129964 (100%) |

Source: GLOBOCAN, 2018

Incidence: is the number of new cases occurring in a given time and geographical area, either as the overall number of cases per year or as the average per 100,000 individuals per year.

Mortality: the number of deaths occurring in a given region and period, and the death rate is the number of deaths per 100,000 individuals per year.

Prevalence = number of cases/ population size

2.2.1 Types of lung cancer

On the basis of histology, lung cancer has two primary types, Non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). This grouping is done on microscopic appearance of the cancer cell. NSCLC is one of major type of lung cancer that causes about 85% of lung cancer and accounts for 15% of lung cancer deaths (Oser et al., 2015). It is a case of lung malignant neoplastic disease in which most of the patients having advanced metastatic stages at diagnosis time (Crino et al., 2010). NSCLC, different from SCLC both histopathologically and clinically. It is further categorized into major three subtypes: adenocarcinoma, carcinoma of squamous cells (squamous-cell carcinoma) and carcinoma of large cells (large-cell carcinoma) (Zappa and Mousa., 2016). Adenocarcinoma is among them the most prominent form of lung cancer in both smokers and non-smokers accounts for nearly 40% of all lung cancers (Sorensen et al., 1993; Couraud et al., 2012). Adenocarcinoma originates from the outer part of the lung's glandular cells and these cells help to secrete mucus that protect against foreign particles entering the lung. Until metastasizing, adenocarcinoma grows slowly and is usually diagnosed, making prognosis greater than most lung cancers (Simon, 2016). This is attributed to variations in some genes, such as anaplastic lymphoma kinase (ALK), epidermal growth factor receptor (EGFR), and Kirsten RAt viral oncogene homologous sarcoma (K-RAS). Mutations in K-RAS are mainly smoking-related and account for around 25 % of cases of adenocarcinoma, whereas ALK and EGFR are not smoking-related (Markman et al., 2018). Adenocarcinoma treatment options largely depend on the stage of cancer.

Squamous-cell carcinoma (SCC): The other type NSCLC, it originates from the squamous cells in the airway epithelial cells and mainly caused by cigarette smoking, pipes, and cigars (Kenfield et al., 2008). It is also known as epidermoid carcinoma and constitutes about 30-35% of all lung cancers and are mostly due to heavy smoking (Rubin and Reisner, 2009). Squamous

cell carcinoma encountered mostly male smokers and increases after long time disclosure to smoking (Molina et al., 2008). The characteristic features of this disease are loss of the ciliated columnar epithelium, basal cell hyperplasia, low columnar epithelium without cilia followed by metaplasia. If the exposure to causative agents continues there is transformation of glandular epithelium to squamous epithelium (Fischman et al., 2014). They may spring up to large sizes and form cavities in the lung (Molina et al., 2008). Due to the constant flow of body fluids, such as blood and lymph, which carry cancer cells to surrounding areas, this form of lung cancer has a great opportunity to spread through the lungs to other parts of the body (Merina et al., 1977).

Large cell carcinoma is a least common type of all NSCLC comprising 5-10% of all lung cancers. It normally takes place between the lymph nodes and the chest wall in the central part of the lungs (Brambilla et al., 2004). Large cell carcinomas histologically demonstrate the areas and layers of large cells with vesicular nuclei, prominent nuclei, and small quantities of cytoplasm (Zander and Farver, 2018). This type of lung malignant neoplastic disease is strongly linked to smoking (Muscat et al., 1997).

Around 10-15% of all forms of lung cancer are small-cell lung cancer. In 1926, Barnard for the first time described SCLC as a tumor of bronchus having round cell carcinoma (Barnard, 1926). It is an adverse, lethal and broadly metastatic type of lung cancer that originates from neuroendocrine cells and due to its appearance this carcinoma is recognized as “Oat Cell Carcinoma”. Under microscope, this cancer looks small and grayish and oval-shaped cells, thus similar to an oat (Demedts et al., 2010; Kumar et al., 2017). These cancer cells are extremely fatal with thin cytoplasm and rough chromatin. It has an early metastatic effect in the parenchyma of the lung which usually includes hilar and lymph nodes (Fischman et al., 2014). SCLC associated deaths are estimated to be 2.5 lakh persons annually with five year survival rate about 7% (Rudin and Poirier, 2016). The threat of SCLC is more in males as compared to females because males usually smoke more than females. Another explanation for this is that more men works in factories and exposed to different contaminants such as asbestos, radon and uranium (Demedts et al., 2010; Fischman et al., 2014). The most common risk factor for SCLC is smoking, while other causes include occupational contact to harmful chemicals, a family history and second-hand smoke (Hubaux et al., 2012). Over 95% of patients develop smoking related SCLC and this hazards increasing with the number of cigarettes consumed each day and

the length of smoking. Smoking and other chemicals cause lung damage by affecting the lung epithelium and finally cause SCLC (Tan et al., 2018). The SCLC development also leads certain “Para neoplastic syndromes” associated with it because of the excessive discharge of certain hormones (Bunn and Carney, 1997).

The common treatment for all types of lung cancer involves surgery, chemotherapy, immunotherapy and radiation therapy (Hussain et al., 2019). The chemo and radiation therapy combination improves the survival rate and also extend the patient life, as they together work to stop the growth and kill the cancerous cells. Chemotherapy works in such a way that it preventing cancer from metastasizing and radiation terminate the cancer cells by its high-energy particles. SCLC spreads very fast because of which operation is not recommended as incision from one position alone will not be enough to remove the cancer (Fischman et al., 2014). The treatment alternatives for adenocarcinoma are largely dependent on stage of cancer. If it is still present, and has not evolved, then the best option is surgical resection. The chemotherapy is often used to avoid the growth first and then to resect the tumour surgically. Consequently, chemo-radiation will be used to avoid the bed cover and kill the cancer cells, followed by surgery, necessary if cancer has spread into the lungs (Markman et al., 2018).

2.2.2 Lung cancer causes

Cigarette smoking is assumed to be the world’s prominent root of deaths related to lung cancer (Freedman et al., 2008). It is reported that tobacco use is a root cause for about 22% deaths globally (Jemal et al., 2011). The lung cancer incidence continues to be high each year due to increasing cigarette smoke intake (World Health Organization, 2003). It is reported that there are over 7,000 chemicals in cigarette smoke at least more than 250 are found to harmful including hydrogen cyanide, carbon monoxide, and ammonia (NTP, 2016). More than 69 chemicals are known to cause cancer are listed in **Table 3**. Among all these chemicals present in cigarette the polycyclic aromatic hydrocarbons (PAHs) plays an essential part in lung tumorigenesis (Hoffmann et al., 2001).

Table 3: Carcinogens present in cigarette smoke

| S. No | Type |
|-------|----------------------------------|
| 1. | Polycyclic aromatic hydrocarbons |
| 2. | Aromatic amines |
| 3. | Tabacco specific nitrosamines |
| 4. | Heterocyclic aromatic amines |
| 5. | Aldehydes |
| 6. | Aza-arenes |
| 7. | Vinyl chloride |
| 8. | Acetaldehyde |
| 9. | Arsenic |
| 10. | Formaldehyde |
| 11. | Arsenic |
| 12. | Benzene |
| 13. | Polonium-210 |
| 14. | Cadmium |
| 15. | Chromium |
| 16. | 1, 3–Butadiene |

Source: Department of Health and Human Services (HHS), 2010

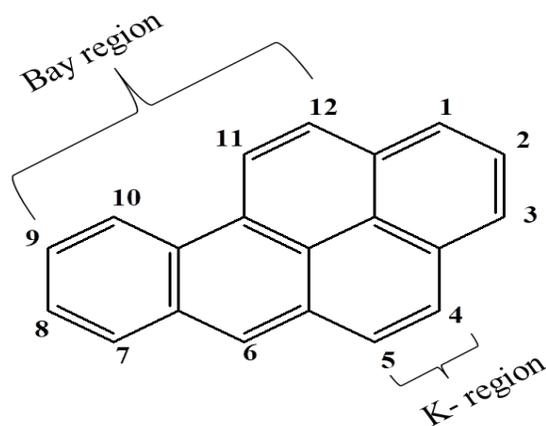
2.2.3 PAHs (Polycyclic aromatic hydrocarbons)

Aromatic hydrocarbons that have two or more fused benzene rings are PAHs, formed into the atmosphere by an incomplete burning of fossil fuels and biomass (Phillips, 2002; Yu et al., 2019). They are a major threat for public health as some of these compounds are reported to possess carcinogenic, mutagenic and teratogenic effects on humans (Ellard et al., 1991; Yu et al., 2019). PAHs are one of the major constituents that play a critical role in the genesis of lung cancer (Hoffmann et al., 2001). They are produced by both natural and human activities in the environment. The natural sources are land fires, volcanic eruptions and oil seeps. However, the sources of PAHs generated from human activities are tobacco smoking, fossil fuel combustion, wood and coal tar; garbage; using lubricating oil and oil philtres; incineration of industrial solid

waste; fuel spills and discharges (Kaushik and Haritash, 2006). Some cancerous PAHs present in the tobacco smoke are as benzo (a) pyrene [B(a)P], benzo [j]- fluoranthene [B(j)F], benz-[a] anthracene [B(a)A], benzo [b] fluoranthene [B(b)F], dibenzo [a,i] pyrene [D(ai)P], dibenz [a,h] anthracene [D(ah)A], dibenzo[a,e]pyrene [D(ae)P], benzo[k]fluoranthene [B(k)F], 5-methylchrysene (5MC) and indeno [1,2,3-cd] pyrene [I (cd)P] (Ding et al., 2007).

2.2.3.1 B(a)P (Benzo(a)pyrene)

Benzo (a) pyrene [B(a)P], a molecule with 5 fused benzene rings, is an important member of polycyclic aromatic hydrocarbon family with molecular formula $C_{20}H_{12}$ as depicted in **Fig. 5**. It exists as pale yellow solid with an aromatic smell and relative molecular mass of 252.31 (IARC, 2010). The basic structure is believed to be responsible for carcinogenicity of PAHs are a bay region and fjord (hindered-bay) region (Marston et al., 2001). This bay region is a complex ring structure composed of a saturated angular benzene ring fused with an aromatic ring into a polycyclic aromatic hydrocarbon (Lehr et al., 1977; Wislocki et al., 1978). In this region the radical formation takes place easily, while detoxification and conjugation are blocked. The B(a)P epoxides formed on bay-region are more prone to nucleophilic attacks, such as DNA and attract carbonium ions more freely than non-bay region epoxide (Pelkonen and Nebert, 1982). The B(a)P metabolite, such as B(a)P 7,8-diol-9,10-epoxide (BPDE) has a 10-position epoxy ring used with the guanine N2 position to form DNA adducts. At position 4 and 5 the carbon atoms are having high electron density and this region is also known as K-region.



Benzo(a)pyrene

Fig. 5. Structure of benzo(a)pyrene (C₂₀H₁₂) showing bay and K-region reactive sites.

It is a strong pro-carcinogen and is one of the major component of cigarette smoke with the ability to induce lung tumors in all of the studied species, such as rat, mice, guinea-pig, hamster, rabbit and monkey (IARC, 1973; Ravindra et al., 2008; Kalabus et al., 2012). The International agency for research on cancer (IARC), graded B(a)P as group 1 carcinogen, because of its carcinogenic and mutagenic characteristics (IARC, 2010). The PAHs that are low in molecular weight, such as phenanthrene, are present almost exclusively in the gas phase, whereas heavier PAHs like B(a)P are almost entirely adsorbed into particles (Ravindra et al., 2008). The worldwide public can be exposed to B(a)P through various routes such as dermal exposure occurs by coming in contact with soot, tar, or crude petroleum containing materials, by intake of foods like fried chicken, grilled and smoked dried beef, potato chips, and also by inhalation of cigarette smoke and diesel exhaust, polluted air, contaminated food and water ingestion (Sinha et al., 2005; Swauger et al., 2002; HSDB, 2012). Among the multiple sources of B(a)P exposure, high content was found in tobacco smoke (20-40ng/cigarette) and grilled beef (58.8-66.6ng/g) as depicted in **Table 4**. The carcinogenic compounds are divided into genotoxic and non-genotoxic carcinogens on the basis of their mode of action (Ashby, 1992; Lima and Van der Laan, 2000). B(a)P possesses both genotoxic and non-genotoxic properties, direct DNA damage after metabolic activation and induced breakage of DNA and formation of DNA adducts (Van Delft et al., 2004; Kasala et al., 2015). Moreover, non-genotoxic carcinogens alter the balance between growth and death through various pathways such as cell-cell communication, modulation of metabolizing enzymes, stimulation of oxidative stress, DNA methylation, DNA repair and many more (Williams, 2001; Hernandez et al., 2009). Various routes of B(a)P administration like oral, subcutaneous, dermal and inhalation were used in the mouse model to induce lung tumorigenesis (Conaway et al., 2005; Yan et al., 2005; Rajendran et al., 2008; Manna et al., 2009; Yeo et al., 2017). The B(a)P installation revealed its effect after a single intraperitoneal (i.p) dose (100mg/kg, BW) leads formation of tumors in 20-22 weeks (Yan et al., 2005), Orally (50mg/kg, for four weeks twice a week) mice develop tumors in 16-18 weeks (Rajendran et al., 2008).

Table 4: Concentration of B(a)P in different sources

| Sources | B(a)P concentration |
|---------------------|-----------------------|
| Tobacco smoke | 20-40ng/cigarette |
| Fired chicken | 5.3-5.6ng/g |
| Exhaust from diesel | < 5 ng/m ³ |
| Oils of sesame | 0.36 ng/g |
| Grilled beef | 58.8-66.6 ng/g |
| Chips from potato | < 4.1 ng/g |
| Smoked died beef | 5.5 ng/g |

Source: Uno and Makishima, 2009; B(a)P (Benzo(a)pyrene)

2.2.3.2 Mechanism of B[a]P induced lung cancer

B(a)P is a pro-carcinogen that becomes complete carcinogen after metabolism in animals and cause changes in some essential genes through binding with DNA (Kasala et al., 2015). It is metabolized by both phases of enzymes such as phase I and II that forms a chain of arenic oxides, dihydrodiols, quinones, phenols and their sulphate, glutathione and glucuronide polar conjugates (Osborne and Crosby, 1987). CYP 1A1 and CYP1B1 essential phase I metabolic enzymes that are engaged in the bio-activation of B(a)P, however metabolic enzymes of phase II involved in the detoxification cycle are GST (glutathione -S-transferase) and UDP-GT (uridine diphosphate glucuronosyl transferase) (Spink et al., 2002; Baird et al., 2005). It is possible to metabolize B(a)P into four separate stereoisomers of B(a)P-7,8-diol-9,10-epoxide (BPDE) as depicted in **Fig. 6**. Among them (+)-anti-isomer is most powerful tumour initiator and can bind to DNA at nucleotide guanine N₂ location forming a highly carcinogenic DNA (+)-trans-anti-BPDE-N₂-dG adduct (Baird and Ralston, 1997). These DNA adducts are highly resistant to the main repair mechanism (nucleotide excision repair, NER) for B(a)P-induced bulky adducts and large lesions (Cheng et al., 2000). The down-regulation of NER genes such as excision repair cross-complementation group 1 (ERCC-1) is related to development of

pulmonary cancer (Cheng et al., 2000; Gillet and Schärer, 2006). Therefore, if these adducts are not eliminated with DNA replication and translational synthesis that causes transversions of G→T base pairs (Zhang et al., 2002). It is understood that lung carcinogenesis involves BPDE mediated G→T mutations in p53 or Kras genes (Pfeifer et al., 2002). The changes in the proto-oncogene such as Kras2 and anti-oncogene p53 and p16 were noticed in both mice and human lung tumors (You and Bergman, 1998). In pulmonary tumors of mice induced with B(a)P, the anti-B(a)P-7,8-diol-9,10-epoxide N2-deoxyguanosine adducts showed at codon 12, G→T and G→A mutations in the K-Ras gene (Mass et al., 1993).

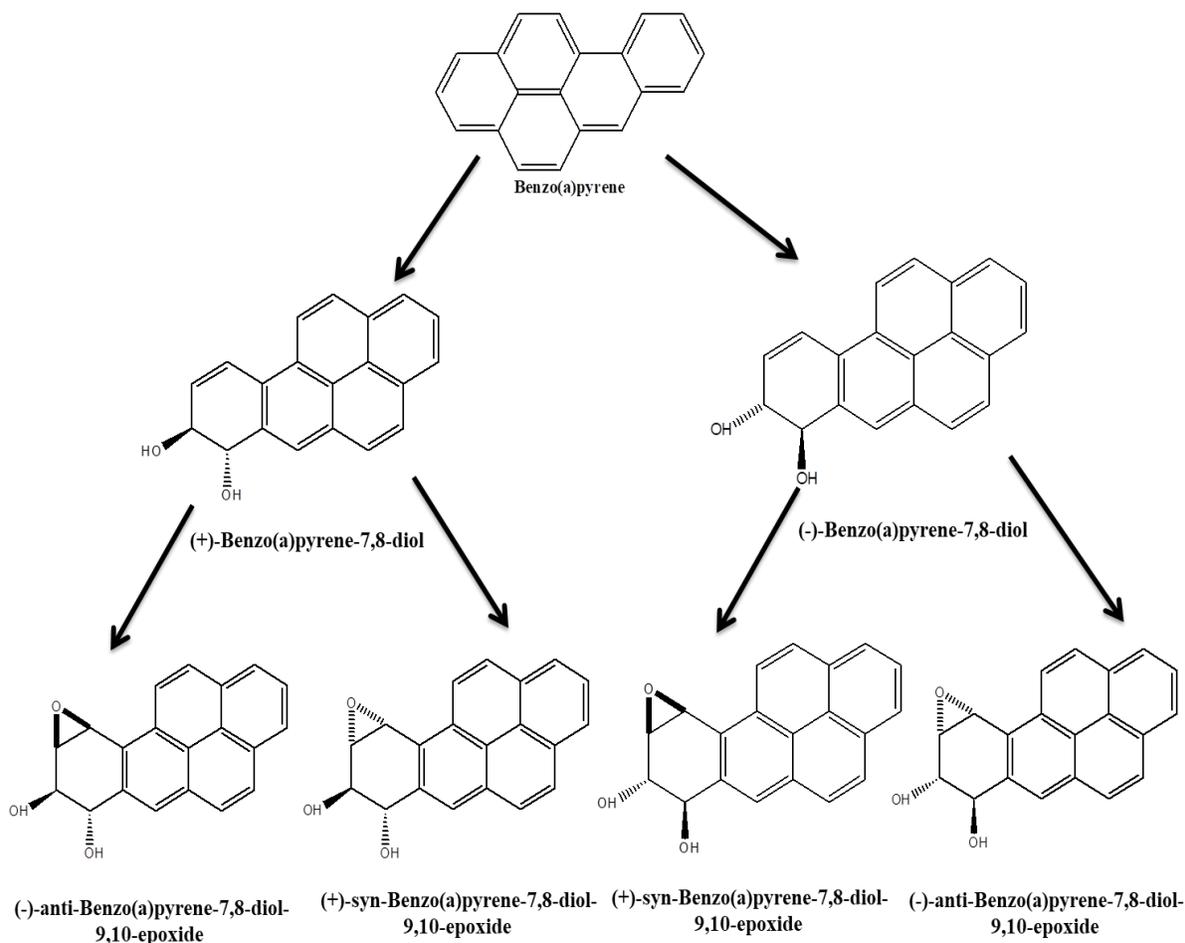


Fig. 6: Benzo(a)pyrene metabolism into its diols and diol epoxides (Baird and Ralston, 1997), BPDE [benzo(a)pyrene -7,8- dihydrodiol-9,10-epoxide].

In B(a)P detoxification process, CYP1A1 and CYP1B1 add an epoxy group to B(a)P, causing formation of benzo(a)pyrene-7,8-epoxide and B(a)P-9,10-epoxide, further transformed to GST catalyzed benzo(a)pyrene-glutathione conjugates. Such epoxides may also change to benzo(a)pyrene-7, 8- diOH or B(a)P-9,10-diOH by epoxy hydrolase and to benzo(a)pyrene - 3-phenol by non-enzymatic means. The UDP-GT and sulfonyl transferase (SULT) altered the benzo(a)pyrene - 3 - phenol into water soluble conjugates as shown in **Fig.7**. The dihydrodiols can be converted to water soluble substrates or transformed to BPDE by CYP1A1 or CYP1B1 which forms DNA adducts (Baird et al., 2005; Jin et al., 2006). Active transport across the membrane is the final step of B(a)P metabolism (Hoffmann and Kroemer, 2004). There are some ATP binding transporter proteins that can take benzo(a)pyrene, other chemicals and their metabolites across the membrane. B(a)P discharge into human breast cancer cells is interfered with by the P-glycoprotein and protein resistance to breast cancer may also deliberately carry B(a)P glucuronide, glutathione and sulphate conjugates (Ebert et al., 2005; Srivastava et al., 2000). Alterations in these proteins interfere with the compounds carcinogenicity, such as decreased BPDE-glutathione conjugate removal, resulting in increased BPDE-DNA adduct generation (Gillet and Scharer, 2006).

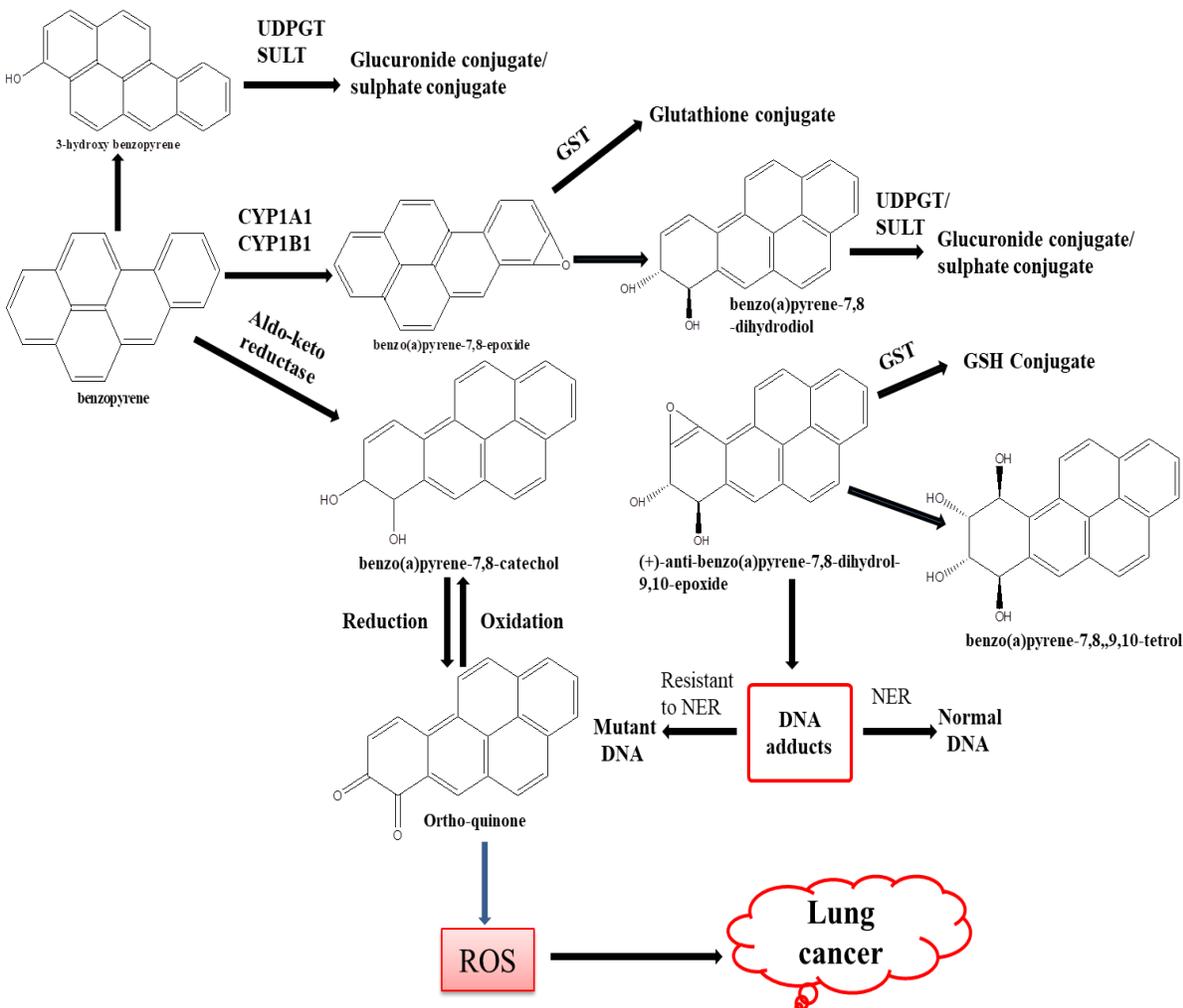


Fig. 7: Mechanism of B(a)P by ROS and DNA adducts formation that finally cause lung cancer (Kasala et al., 2015). Uridine diphosphate glucuronosyl transferase (UDPGT), sulfotransferase (SULT), Reactive Oxygen Species (ROS), Glutathione (GSH), Nucleotide Excision Repair (NER) and Glutathione-S-transferase (GST)

In addition to general pathways, it is also possible to metabolize B(a)P to form O-quinones by aldoketo reductases and these O-quinones may also undergo redox cycling to generate a high amount of free radicals that are responsible for oxidative stress and greatly increased peroxidation of lipids (Penning et al., 1999). The increment for lipid peroxidation levels and decline in antioxidant defense system are highly prone to oxidative damage and could be reason for cancer. Increased levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in mice following

B(a)P administration showed the capacity of B(a)P to inflict oxidative damage (Garg et al., 2008). In addition to DNA damage, ROS also cause alterations in tumor micro-environment that boosts tumor progression. In several biological processes ROS acts as signaling intermediates such as the activation of nuclear factor kappa B (NF- κ B) by increase in ROS level, plays an significant role in tumor development (Lee et al., 2013). B(a)P uses an AhR (aryl-hydrocarbon receptor)- mediated signal transduction to induce tumor growth and plays a vital part in B(a)P-induced carcinogenesis (Ross et al., 1995). As it is a lipophilic compound, once it enters the body it easily crosses the cell membrane and in cytoplasm it gets attached to AhR that forms chaperone protein complex such as heat shock protein 90, XAP2 and P23 (Rayes et al., 1992). Binding B(a)P to AhR shows how these complexes are activated translocated into the nucleus. These complexes bind to the TNGCGTC consensus sequence of xenobiotic response elements (XREs) of various promoter region genes such as CYP1A1, CYP1A2, CYP1B1, UDP-GT, GST, and quinone oxidoreductase after AhR nuclear translocator (ARNT) heterodimerization and induce the expression of different genes (CYP1A1 and 1B1) which plays an significant character in benzo(a)pyrene metabolism (Gelboin, 1980). It was reported that an important role was played by AhR in various cellular processes such as cell-cycle regulation, inflammation, proliferation of cells and apoptosis (Nebert et al., 2000). The general B(a)P pathway mediated initiation and progression of the lung tumor are shown in **Fig. 8**.

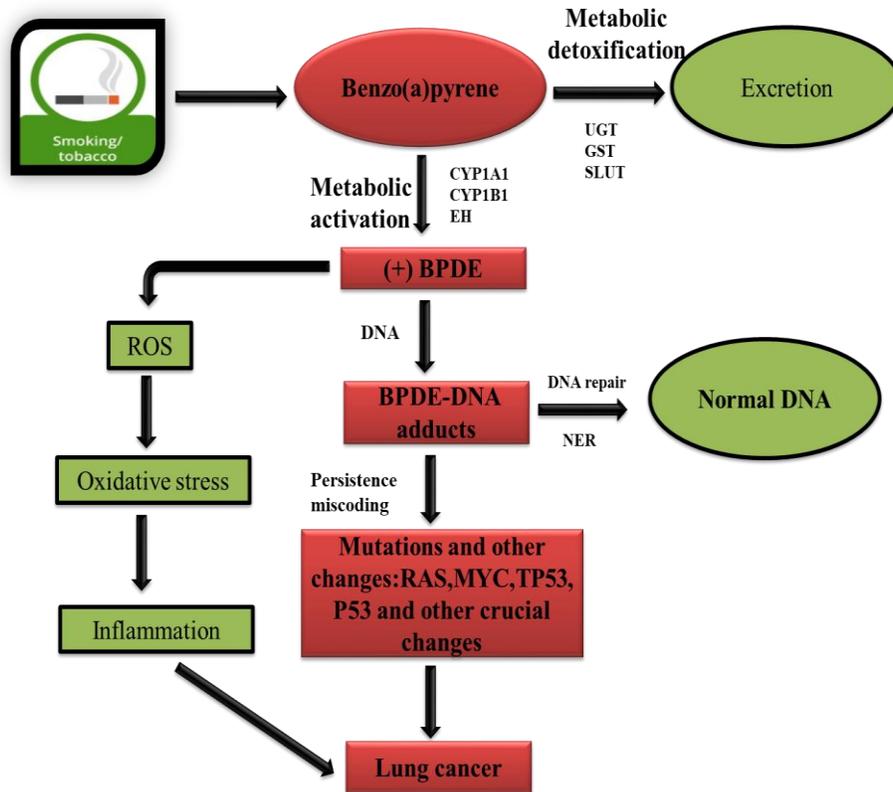


Fig. 8 : Systematic presentation of B(a)P pathways involved in lung cancer (Kasala et al., 2015). Epoxide hydrolase (EH), Uridine 5'-diphospho-glucuronosyltransferase (UGT), Glutathione S-transferase (GST), Sulfotransferase (SULT), Benzo(a)pyrene -7, 8-dihydrodiol-9, 10-epoxide (BPDE), Reactive oxygen species (ROS), Nucleotide excision repair (NER).

2.3 Approaches of lung cancer Treatment

Approaches for treatment of lung cancer includes surgery, radiotherapy and chemotherapy. One of the main options for treating patients diagnosed with lung cancer is surgery. Sometimes it is the only treatment required, while sometimes it is combined with chemotherapy and/or radiation therapy (Howington et al., 2013). In chemotherapy, the chemotherapeutic drugs such as cisplatin, etoposide, paclitaxel and carboplatin are used to destroy cancer cells. Lung cancer patients are treated in order to reduce hostile effects and increase the survival rate. For NSCLC patients the first-line therapy is the cytotoxic combination chemotherapy, which may be effected by various factors such as patients age, histology, performance status and comorbidity (Ramalingam and Belani, 2008). Chemotherapy is one of the key therapeutic approaches that can be used as an efficient treatment technique for cancer, but various studies have shown that

this method is having number of drawbacks; it affects the normal cell division and replication (Wu et al., 2008). The chemotherapy for advanced NSCLC was observed to be unsuccessful or unnecessarily toxic, but meta-analyses reports revealed that as compared with supportive care, chemotherapy displayed a modest increase in the persistence of NSCLC patients (Grilli et al., 1993; Marino et al., 1994). Radiation therapy is carried out by employing high-energy beams to destroy the cancer cells by damaging their DNA. It is applied before surgical operation, to make a tumour smaller and easier to get rid of, most often in combination with chemotherapy. Irradiation with or without chemotherapy may be performed after surgery, to the destruction of cancer cells that may still be present following surgery (ASRO, 2009). In spite of advances in treatment and diagnosis of lung cancer, it was reported that survival rate remains low and lung cancer proved to be difficult to control with surgical and therapeutic approaches. The usual therapies like chemotherapy, radiation therapy or their combination remain unsuccessful to make a major impact on survival due to lack of reliable biomarkers for early stage diagnosis of the diseases. These therapies are found to be ineffective for lung cancer because the recovery of the patients is very less. The role of various conventional lung cancer therapies like chemo, targeted, immuno and radiation therapy have induced thoughtful side effects. In some instances, due to immunosuppression and organ failure, patients may fail before they retrieve (Temal et al., 2006).

2.4 Cancer chemoprevention

Dr. Michael B. Sporn first coined the term chemoprevention (Sporn, 1993). Chemoprevention is the use of non-cytotoxic nutrients or pharmacologic compounds that can stop or inverse the process of cancer (Sporn, 1976). The goal of chemoprevention of lung cancer is to uncover those substances that can block the growth of cancer. This can be a credible and cost effective method that reduces the death rate by reduction in premalignant incidents before the occurrence of pathological changes (Kasala et al., 2015). Lung carcinogenesis is best example for chemoprevention, as it is usually diagnosed with relatively slow growth rate and development in elderly population (Gomperts et al., 2011). Instead of significant advances in lung cancer therapy, including chemotherapy, radiotherapy, targeted therapy and immunotherapy the average survival rate is lowest in lung cancer (Niyazi et al., 2011). There is an immediate need to implement efficient cancer prevention and treatment approaches with less or no toxic effects

(Shu et al., 2013). One of the approaches may be use of botanicals in the form of beverages, vegetables, fruit and other nutritional requirements that can inhibit, block, suppress the progression of neoplastic cells to cancer, that can be a reasonable and cost-effective method (Yan et al., 2006). Several studies reported that hazards of cancer can be influenced by dietary factors (Dull and Davies, 1991; Wattenberg, 1992). The various plants, herbs, beverages, vegetables and spices used in ethnic and traditional medicine were found as one of the major source of discovery and production of chemopreventive drugs for cancer (Abdullaev, 2001; Kaefer and Milner, 2008). The capability of different dietary phytochemicals as a chemopreventive substance against cancer is increasingly valued. Various epidemiological reports showed that the habit of eating fruits and vegetables is associated with decreased threat of lung cancer occurrence (Block et al., 1992s; Keith, 2009). An ideal chemopreventive agent must have the following properties as shown below in **Fig. 9**.

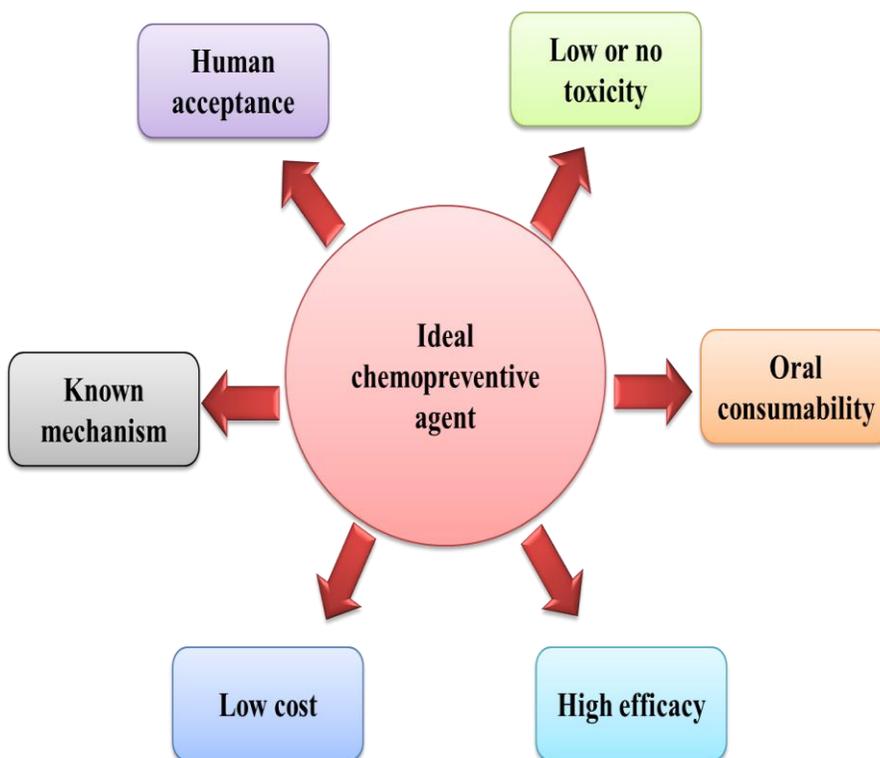


Fig. 9: Properties of an ideal chemopreventive agent (Mukhtar and Ahmad, 1999)

Chemopreventive agents such as man-made or naturally occurring phytochemicals that demonstrated various types of cellular effects as depicted in **Fig.10**. The major challenges to

discover the mechanism of chemoprevention by various plant extracts focused on; antioxidant and free radical quenching ability, effects on detoxification enzymes, targeting the activated metabolites of different carcinogens, prevention against genotoxicity, inhibition of tumor initiation and promotion (Katiyar and Mukhtar, 1996). These agents work against B(a)P, triggered lung cancer by targeting specific cellular processes like obstructing the production of B(a)P metabolites by hampering the activity of phase I enzymes, provoking phase II enzymes, and boosting DNA repair system (Rajendran et al., 2008). These agents also showed their effect by quenching oxygen radicals, reducing the metabolism of glycoproteins and polyamine or by inhibiting certain signal transduction pathways, reduction in cell proliferation and increment in apoptosis, thereby inhibiting cell invasion, angiogenesis and metastasis (Kuroda and Hara, 1999; Anandakumar et al., 2008; Garg et al., 2008; Yang et al., 2009). The molecular pathology of lung cancer is a complicated mechanism because it involves several genes and additional environmental factors like inflammation and dietary substances (Gompers et al., 2011; Smith et al., 2006). The significance of dietary phytochemicals that are having chemopreventive property is highly enhanced. Various plant phytochemicals were reported to have anticancer activity against B(a)P initiated pulmonary cancer while targeting the above mentioned pathways that plays a part in cancer development (Garg et al., 2008; Rajendran et al., 2008). The polyphenols from green tea affect multiple targets that may be the reason for its cancer chemopreventive potential. The various dietary phytochemicals/whole extracts and beverages that act as chemopreventive agents against lung tumorigenesis are given in the **Table 5**:

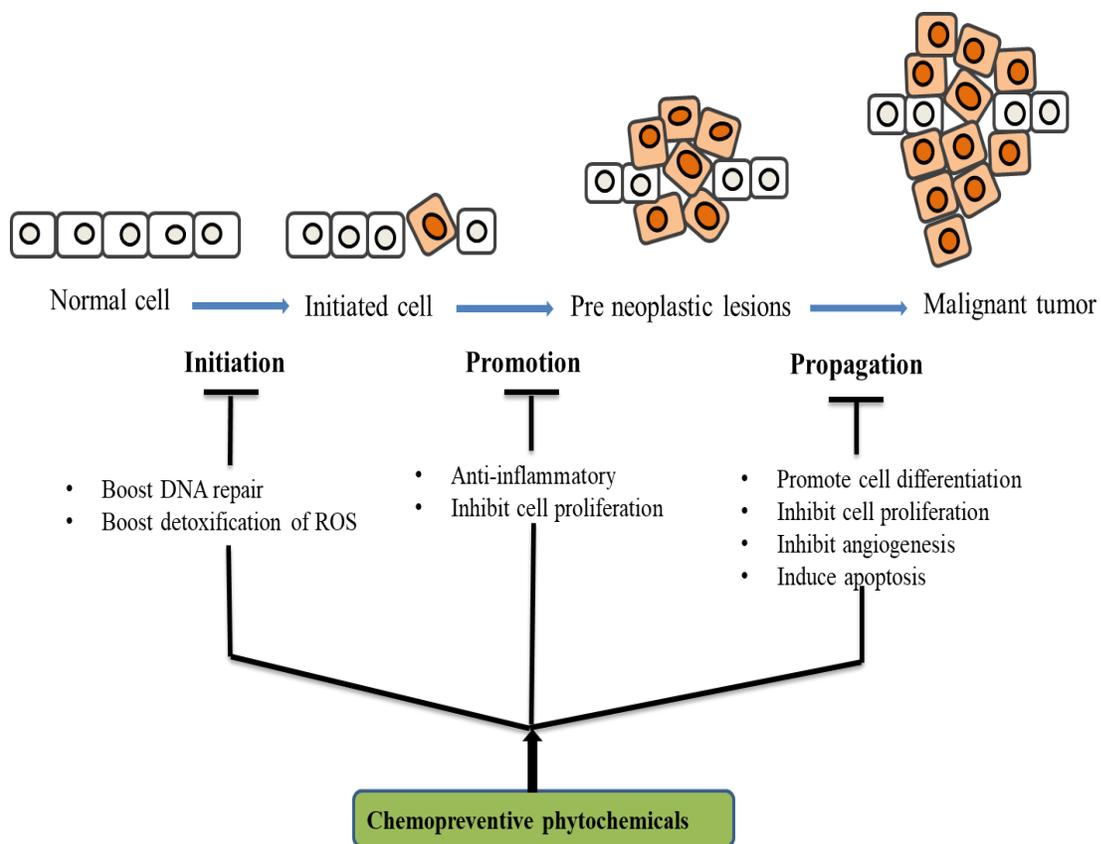


Fig.10: Carcinogenesis a multi stage process and role of chemopreventive effect of phytochemicals (Surh, 1999)

Table 5: Studies carried in animal models by applying dietary phytochemicals/whole extracts/beverages or other herbs against B(a)P or other chemical induced lung cancer.

| S. No | Dietary Phytochemicals /whole extract | Animal species/age/ time duration. | Dose of phytochemicals/ drugs | Dose/route of administration/ frequency of dosing | Mode of action | References |
|-------|---|---|--|---|---|----------------------|
| 1. | Biochanin | Non-inbred swiss-Webster mice of less than 24 hours old. 9 week experiment. | Biochanin (0.125mg/0.1ml of DMSO), three times/week for 6 weeks. | B(a)P (0.5mg in 1% aqueous gelatin), a subcutaneous injection with 0.02 ml of suspension in the scapular region | <ul style="list-style-type: none"> • Significant decreases in body weight and relative lung weight, respectively. • Decrease in lung tumor occurrence and multiplicity. | Lee and Seo, 1991 |
| 2. | Green tea and licorice. | Female A/J mice of 6-8 week old. 35 week study. | Green tea water extract (1.2 %) and licorice (1 %), as a single source of drinking water 2 weeks before administration of B(a)P and continued until the end of the experiment. | B(a)P (100 mg/kg, BW) in 0.2ml corn oil p.o., total four times at 2 week interval. | <ul style="list-style-type: none"> • Green tea and licorice reduced the lung tumor occurrence and number. | Wang et al., 1992 |
| 3. | Green tea (GT) and Polyphenolic fraction (PF) | Female A/J mice of 6 week old. 28 week study. | Aqueous extract of green tea (2.5%) and Polyphenolic fraction (0.2%) sole source of drinking water 15 days prior to that of B(a)P administration | B(a)P (2mg/animal), administered orally repeated once every 2 weeks till 6 weeks. | <ul style="list-style-type: none"> • GT and PF reduced lung tumor incidence and multiplicity. • Increased the enzymes for phase II detoxification (GST and QR) | Katiyar et al., 1993 |

| | | | | | | |
|----|---|--|---|---|--|----------------------|
| | | | and continued up to 1 week after the last dose of B(a)P. | | <ul style="list-style-type: none"> • GT was found to be more effective than PF. | |
| 4. | The polyphenolic fraction (PF) isolated from green tea. | A/J female mice of 6 weeks old. 28 week study. | Polyphenolic fraction (5 mg/kg, BW), dissolved in 0.2 ml cotton seed oil and administered orally 30 min earlier to that of B(a)P dose (repeated once every 2 week and total of three doses) | B(a)P (2 mg/animal in 0.2 ml cotton seed oil) taken by mouth and repeated every 2 weeks for a total of 3 doses. | <ul style="list-style-type: none"> • Lowering the number of pulmonary tumors per mouse. | Katiyar et al., 1993 |
| 5. | Green tea | Female A/J mice of 6 week old. 16 week study. | Green tea solids in drinking water (0.1, 0.2, 0.4, and 0.6%) given as a sole source of drinking fluid starting 2 days after NNK injection upto the termination. | NNK (100 mg/kg, BW), a single intraperitoneal dose. | <ul style="list-style-type: none"> • Decline lung tumor number, adenoma multiplicity and angiogenesis. • Increases in the apoptosis index. • Lower concentration of green tea (0.1, 0.2 and 0.4) did not show a significant inhibition. | Liao et al., 2004 |
| 6. | Deguelin and silibinin | Female A/J mice of 6 week old. 22 week study. | Deguelin (5 or 10mg/kg, BW) intragastrically 5day/week for 22 weeks. Silibinin (0.05% and 0.1%) mixed with diet | B(a)P (100mg/kg, BW), a single i.p, dose in 0.2ml of tricapyrin. | <ul style="list-style-type: none"> • No signs of gross toxicity or loss of body weight in both deguelin or silibinin treated animals. • Deguelin reduced the lung tumor multiplicity and | Yan et al., 2005. |

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| | | | | | <p>tumor load.</p> <ul style="list-style-type: none"> • Whereas, silibinin treatment did not demonstrate any significant efficacy on either tumor multiplicity or tumor load. | |
| 7. | Polyphenon E (standardised preparation of polyphenol containing 65% EGCG for green tea) and caffeine | Female A/J mice of 4-6 weeks old. 52 week study. | Polyp E (0.5%) administered to the mice until the end as the sole source of drinking fluid. | NNK (103 mg/Kg, BW), a single i.p, dose. | <ul style="list-style-type: none"> • Reduction in number of lung tumors and tumor volume by both poly E and caffeine. • Decrease in tumor incidence by poly E while caffeine did not influence the multiplicity or incidence of adenoma. • Decrease in proliferation index and increase in apoptotic index both in poly E and caffeine treated animals. | Lu et al., 2006 |
| 8. | Polyphenon E (poly E), Rapamycin (RM) and Red ginseng (RG). | Female A/J mice of 6 weeks old. 26 week study. | Polyphenon E (concentrations: 0.5%, 1.0%, 1.5% and 2.0%), in diet given one week after B(a)P initiation. | B(a)P (100mg/Kg, BW) in 0.2ml of tricapyrin | <ul style="list-style-type: none"> • Higher dose of poly E (2.0%) revealed a significant decline in lung tumor number and tumor | Yan et al., 2006 |

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| | | | <p>Red ginseng (2 or 10mg/ml) in drinking water given before one week of B(a)P dose.</p> <p>Rapamycin (2mg/Kg, BW), i.p, 5 times a week for 14 weeks given twelve weeks after B(a)P introduction.</p> | | <p>load, whereas lower doses found to be less effective in decreasing tumor multiplicities.</p> <ul style="list-style-type: none"> • The higher dose of RG (10mg/ml) decreased tumor number and tumor load. Whereas, decrease in tumor size in both doses. • RM treatment also showed a decrease in tumor number and tumor volume as compared to control group. | |
| 9. | <i>Syzygium aromaticum</i> L. (clove) | Strain A mice of 24-48h old. 26 week study. | <p>Aqueous infusion of clove (2g/100ml) and this infusion was given orally at a dose (100µl/mouse/day) from week 5 of the injection of B(a)P and continued until the end of the experiment.</p> | B(a)P (0.2mg in 1% aqueous gelatin), injected subcutaneously with 0.02ml of a suspension in the scapular region. | <ul style="list-style-type: none"> • Decrease in the occurrence of lung tissue hyperplasia, dysplasia and carcinoma at weeks 8, 17 and 26th, respectively. • Cell proliferation inhibition (PCNA) and apoptosis induction. • Upregulation of pro-apoptotic genes (p53 and Bax) and down-regulation of anti- | Banerjee et al., 2006 |

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| | | | | | apoptotic (Bcl-2) and COX-2, cMyc, Hras the growth-promoting genes. | |
| 10. | Quercetin | Swiss male albino mice of (6-8 weeks old). 16 week study. | Quercetin (25 mg/Kg, BW), i.p., dissolved in 0.1% of DMSO for consecutive weeks, twice per week. It was given one week prior to first dose of B(a)P (Pre-treatment) and from the 8th week of B(a)P (post-treatment) induction to the end of the experiment | Dissolved B(a)P (50 mg/Kg, BW) in corn oil, administered orally twice a week for 4 consecutive weeks. | <ul style="list-style-type: none"> Improved the activities of enzymatic (GR, SOD, CAT, GPx, and GST) and non-enzymatic (GSH, vitamin E and vitamin C) antioxidants of lung tissue in both pre and post treatment. Decrease lipid peroxidation (serum and lungs) and biomarker activities (AHH, γ-GT, LDH and ADA) in both pre and post treatment. Reduction in alveolar damage with near normal structure in pre-treatment while as post-treatment showed slight reduction in alveolar damage. Pre-treatment of quercetin was more | Kamaraj et al., 2007 |

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| | | | | | effective as that post treatment. | |
| 11. | A standardized green tea extract ; [Polyphenon E (Poly E)] | Female A/J strain mice of 6-7 week old. 46 week study. | Poly E (1%) in diet. An inhaled dose of DFMO (20 mg/kg, BW), five days a week | A single i.p., injection of B(a)P (100 mg/Kg, BW). | <ul style="list-style-type: none"> The most abundant catechin detected by HPLC analysis in plasma and lung tissue was EGCG. More reduction in surface lung tumor multiplicity and load was found in poly E +DMFO group and no significant reduction in poly E and DMFO. | Anderson et al., 2008 |
| 12. | The Polyphenols of green tea (GTP) and the polyphenols of black Tea (BTP) | Male Swiss albino mice. 28 weeks study. | GTP and BTP (0.1% and 0.2 %) given as a sole source of drinking water. | 20 mg/Kg, BW of DEN given orally once in a week up to 8 weeks. | <ul style="list-style-type: none"> GTP demonstrated higher reduction in total number of lung tumors compared to BTP. Both extracts inhibited the expression of anti-apoptotic gene (Akt) and proliferating gene (Cox-2) and inactivated NF-kB by blocking phosphorylation and degradation of inhibitor of kappa B (IκBα). | Roy et al., 2010. |

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| 13. | Polyphenon E (Poly E) | Female A/J mice of 6 week old. 20 week study. | Polyphenon E with and without EGCG (15 mg/ml) in 25% ethanol water solution in aerosolized form. | B(a)P (100 mg/Kg, BW), a single i.p., dose in 0.2 ml of tricapylin. | <ul style="list-style-type: none"> • Poly E reduced B(a)P induced lung tumor multiplicity as compared to poly E without EGCG. | Fu et al., 2009 |
| 14. | Tea polyphenols (EGCG and ECG), theaflavins (TF) | Inbred strain A mice of less than 24 hours old. 36 week study. | EGCG, ECG or TF (0.01, 0.004 and 0.02 mg), respectively by i.p., daily. | B(a)P (0.2 mg in 1% gelatin) given a solo subcutaneous injection (0.02ml) at subscapular region. | <ul style="list-style-type: none"> • Treatment of tea polyphenols restricted the lung lesion progression at hyperplastic stage. • On 17, 26 and 36th week, a significant reduction in PI and increase in AI. • On 36th week down-regulation in the expression of growth regulatory genes (H-ras, c-myc, cyclin D1) and anti-apoptotic genes (Bcl-2, p21) and up-regulation in pro-apoptotic genes (p53, Bax and p27) was detected in polyphenol treated groups. | Manna et al., 2009 |
| 15. | Hesperidin and Fisetin | Swiss male albino mice (6-8 weeks old). | Hesperidin and fisetin (25mg/Kg, BW), orally. | Dissolved B(a)P (50mg/Kg, BW) in corn oil, given orally twice a week | <ul style="list-style-type: none"> • Improved both enzymatic (CAT, SOD and GPx) and | Kamaraj et al., 2009 and Ravichandran |

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| | | 16 week study. | It was given one day before the first dose of B(a)P for pre-treatment and continued until the end of the procedure. It was given for post-treatment from the 8th week of induction of B(a)P until 16 weeks. | for 4 consecutive weeks. | <p>non-enzymatic antioxidant (vitamin C, GSH and vitamin E) antioxidant status in pre and post treatment of fisetin and hesperidin.</p> <ul style="list-style-type: none"> • Both pre and post treatment decreased LPO in lung tissues and activities of serum marker enzymes (AHH, GGT and 5'ND) and tumor marker (CEA). • Reduced the lung alveolar/bronchiolar damage and PCNA in pre and post treated groups. • Pre-treatment was found to be more effective than post treatment that may be due its high antioxidant status and inhibitory effect on cell proliferation. | et al., 2011 |
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| 16. | Tea polyphenols (EGCG, EC, EGC and ECG). | Female Sprague-Dawley rats. 16 week study. | 1.2% tea polyphenol solution (200, 27, 19 and 16 mg of EGCG, EC, ECG and EGC). | 3-4 benzopyrene (2mg/0.2ml) intrapulmonary dose at 2 week interval for total four doses. | <ul style="list-style-type: none"> • Lung carcinoma was reduced to 30%. • Increased apoptosis through p53 up-regulation and bcl-2 gene expression down-regulation. | Gu et al., 2013 |
| 17. | Leaf extract of <i>Solanum trilobatum</i> (LEST) | Male Swiss albino mice. 20-25g of BW. 16 week study. | LEST (200mg/Kg, BW). Oral gavage 4 weeks prior to B(a)P dose (pre-treatment). Oral gavage from 12 th week of B(a)P dose and continual upto termination (Post-treatment). | Dissolved B(a)P (50mg /Kg, BW) in corn oil, administered twice a week for 4 consecutive weeks. . | <ul style="list-style-type: none"> • Reduction in lung tumor occurrence, TBARS level (lung and liver) and activities of AHH, γ-GT, 5 ND and LDH (marker enzymes) in LEST pre and post treatment. • The activities of phase I (Cytochrome P450, Cytochrome b5, NADPH Cyt c reductase) and phase II (UDP-glucuronyl transferase, quinone reductase and GSH) xenobiotic enzymes in LEST treatment have been standardized. • Improvement in the activity of LEST treatment with enzymatic antioxidants (SOD, | Venugopal et al., 2014 |

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| | | | | | <p>CAT, GPx and QR) and non-enzymatic antioxidants (GSH, vitamins C and E).</p> <ul style="list-style-type: none"> • Reduction in alveolar damage of lungs and LEST post-treatment revealed slight reduction in alveolar damage. • Pre-treatment was found more effective compared to post treatment. | |
| 18. | Chrysin | Swiss male albino mice (6-8 weeks). 16 week study. | Oral Gavage Chrysin (250 mg /Kg, BW). Pre-treatment began one week before the first dose of B(a)P was given. Post treatment from 8 th week of B(a)P and continued upto termination. | Dissolved B(a)P (50 mg /Kg, BW) in corn oil with oral administration twice a week for four consecutive weeks. | <ul style="list-style-type: none"> • Decrease in lipid peroxides and CEA chrysin pre and post treated mice. • Normalized the enzymatic antioxidant activity (SOD , CAT, GPx and GR) and non-enzymatic antioxidant activity (GSH, vitamin E and vitamin C) • Reduced alveolar damage in a pre-treated group with almost normal architecture and slightly reduced | Kasala et al., 2016 |

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| | | | | | <p>alveolar damage in post-treated animals.</p> <ul style="list-style-type: none"> • Decrease in the expression of PCNA, COX-2 and NF-kB in the lung tissues of pre-and post-treated animals. | |
| 19. | <i>Tinospora cordifolia</i> stems | Male Balb/c mice 25-30g of weight. 22 week study. | Aqueous extract of <i>T. cordifolia</i> (Aq.Tc) (200 mg/Kg, BW) given orally on the alternate days (three times per week) upto 22 weeks. On alternate days (three times a week), arabinogalactan (AG) (7.5 mg /Kg, orally). | B(a)P of 50 mg/Kg, BW, i.p., given twice, over a two weeks interval. | <ul style="list-style-type: none"> • Significant reduction in tumor nodules and tumor mass by Aq.Tc and AG. • The CEA level in mouse blood serum, ctDNA, LDH and TNF-alpha observed to be declined in group (B(a)p + AG) and (B(a)P + Aq.Tc). • Apoptosis was found significantly increased in group [B(a)P + AG] but was much less than the group (B(a)P + Aq.Tc). | Mohan and Koul, 2018 |

Polyphenolic fraction (PF), NADP(H): Quinone reductase (QR), Glutathione S-transferase (GST), NNK (n 4-(methylnitrosamino)-1-(3-pyridyl)-1butanone, Body weight (BW), Green tea polyphenols (GTP), Black tea polyphenols (BTP), Diethylnitrosoamine (DEN), Cyclooxygenase (Cox), Epigallocatechingallate (EGCG), Epicatechin gallate (ECG), Epigallocatechin (EGC), Epicatechin (EC), Theaflavins (TF), Apoptotic index (AI), Dimethyl sulfoxide (DMSO), Superoxide dimutase (SOD), Catalase (CAT), Glutathione

peroxide (GPx), Glutathione -S- transferase (GST), Glutathione reductase (GR), Proliferation index (PI), Glutathione (GSH), Aryl hydrocarbon hydroxylase (AHH), Gamma glutamyl transpeptidase (γ -GT), 5'nucleotidase (5' ND), Lactate dehydrogenase (LDH), Adenosine deaminase (ADA), Lipid peroxidation (LPO), Polyphenon E (Poly E), Difluoromethylornithine (DFMO), High performance liquid chromatography (HPLC) , Green tea (GT), Arabinogalactan (AG), Carcinoembryonic antigen (CEA) , Circulating tumor DNA (ctDNA), Tumor necrosis factor (TNF-alpha), Benzo(a)pyrene [B(a)P], Aqueous extract of *Tinospora cordifolia* (Aq.Tc), Proliferating cell nuclear antigen (PCNA), Leaf extract of *solanum trilobatum* (LEST), Cytochrome P450 (Cyt.P450), Cytochrome b5 (Cyt. b5), NADPH Cyt c reductase (Cyt c.R), UDP-glucuronyl transferase (UDP-GT), Thiobarbituric acid reactive substances (TBARS), Nuclear factor-kappa B (NF- κ B), Rapamycin (RM) and Red ginseng (RG).

2.5 Green Tea

In the world tea is considered as one of the most popular drinks after water, obtained from *Camellia sinensis* plant which belongs to the Theaceae family. In various regions of the world, it is primarily consumed as black, green, white or oolong tea (Chacko et al., 2010). Among all these teas, the green tea has generated much response from the general public and researchers due to its various health promoting properties such as antioxidant, anticarcinogenic, antimutagenic, antihypertensive, antiangiogenic, and antiobesity (Cabrera et al., 2006) **Fig. 13.**

2.5.1 Green tea processing

On the basis of manufacturing process tea is categorized into green, white, black, yellow, oolong and dark teas (Shitandi et al., 2013). Among all these types green, black and white tea is most notable and widely available in the global markets (Feng et al., 2019). Green tea is minimally processed tea and this short processing gives it a lighter flavor and high catechin content than black tea. The various phases involved in green tea manufacturing are given **Fig. 11.**

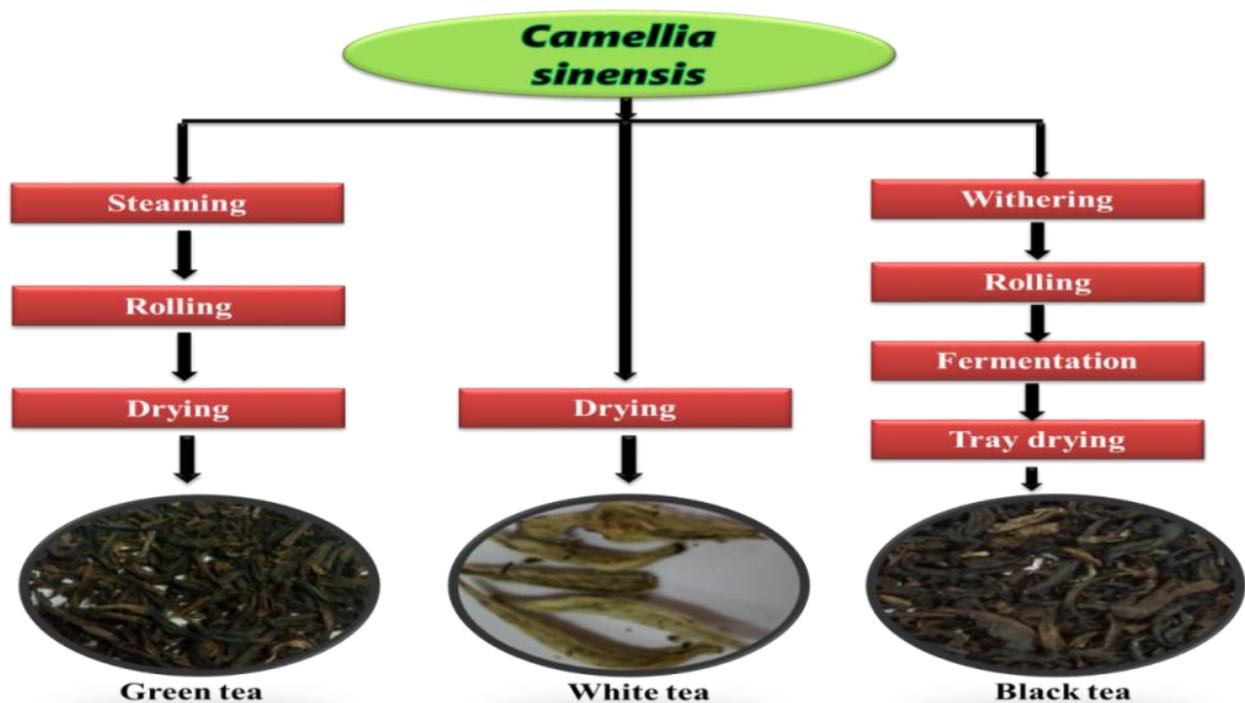


Fig 11: Different steps involved in the manufacturing process of green, white and black tea.

2.5.1.1 Plucking

The first step in harvesting the tender apical shoots (buds and first two to three leaves) by hand or mechanical tea plucker and are carried in open trailers from garden to factory for manufacturing (Lee et al., 2014).

2.5.1.2 Steaming

The plucked tea leaves were subjected to steaming by which polyphenol oxidizing enzymes are deactivated and become biologically inactive due to high temperatures (Yang et al., 2009). In steaming, the leaves are passed along a revolving drum and the hot steam is applied for two minutes, the leaves are then drawn out again (Singh et al., 2014). It is a big step in retaining the performance of green tea because too much steam can spoils the leave and too less can initiate the oxidation process. It is essential for maintaining the appearance, texture and catechin content of the green tea (Xua and Changa, 2008).

2.5.1.3 Rolling

The leaves in this phase are rolled in a rolling machine in order to destruct the leaf tissue, ooze out some sap, and essential oils inside the leaves and shape the final product (Ahmed and Stepp, 2013). The rolling time varies from young to older leaves (10 minutes to 1hour). The young leaves are rolled under low pressure for small time duration as compared to older leaves, thus prevent the leaves from breaking and yellowing (Xu and Chen, 2002).

2.5.1.4 Drying

The rolled tea leaves can be immediately dried at 90-110°C for 5-15 minutes to stop the reactions by lowering the moisture content to get the loose green tea (Wei et al., 2012). The drying is to stop enzymatic reactions and further oxidation, to produce a commodity that can be stored for a long time without any degradation thus maintaining the color and flavor of green tea (Kosisnka and Andlaur, 2014).

The least-processed tea is white tea, in which fresh young leaf buds are plucked and dried immediately after harvesting to prevent oxidation (Rusak et al., 2008). Black tea is a maximally oxidized form of tea and in its manufacturing plucked leaves, shoot tips under the unfolded leaf are also harvested and used (Srikantayya, 2003). After harvesting, in order to lose water, the leaves are placed on the soil or withering groove with continuous ventilation. The goal of withering is lessen the moisture content and to allow the flavor compounds to develop in the tea leaves (Chen et al., 2019). Withering is followed by rolling and then crushing that facilitate the enzymatic oxidation and subsequent condensation of tea polyphenols via a process called fermentation, which contributes to the formation of

theaflavins (theaflavin-3-gallate, theaflavin-3'-gallate and theaflavin-3-3'-digallate) and thearubigins, is responsible for the dark color and bitter taste of black tea (Yang et al., 2002). The tea leaves are dried after fermentation to make the leaves shelf-stable and stops the oxidative processes and maintain the health-promoting properties of polyphenols (Chacko et al., 2010). The manufacturing process thus performs an essential role in the growth of the primary features of each form of tea (Chen et al., 2019). Instead of processing, there are numerous factors that affect the quality of teas such as soil type, climate, racial practices (including ploughing, weeding, fertility management, irrigation, plant safety and picking), cultivars, shapes and methods used in plucking (Chiu, 1989).

2.5.2 Green tea production

In China, tea is cultivated for already thousands of years (Zhen, 2003). Today, the majority of tea generation takes place in India, China, Sri Lanka and several African countries. Total tea production of the world is around 29 lakh tons a year, in which 6 lakh tons of green tea is used up each year. China is the country that produces and drinks the largest green tea and Japan ranks second. The green, black, Oolong, dark and yellow tea total production ratio in China in 2017 was reported to be 67.3%, 11.6%, 10.9%, 9.1%, 1.0%, and 0.2%, respectively. Worldwide the production of green tea is estimated to grow quicker than black tea by 8.2%, this reflects the growth of China, where green tea production is projected to exceed 2.97 million tonnes by 2023. Globally, green tea is also expected to rise by 7.1 percent annually to hit 7 lakh tons by 2023 (Chang, 2015). However, it is calculated that black tea production will increase 4.4 million tons by 2027 from projected value 3.33 million metric tons in 2017 globally. China is the world's biggest producer of green tea and its production is appraised to increase 3.31 million metric tons in 2027 from 1.52 million metric tons in 2017.

2.5.3 Phytochemical composition of green tea

More than 4000 phytochemicals are investigated to be present in tea leaves (Mahmood et al., 2010). They are biologically active secondary metabolites having diverse pharmacological activities, and form an important constituent of diet for humans (Mahmood et al., 2010). Phytochemical profile of green tea leaves is complex and contains carbohydrates (7-25% dry weight) for example glucose, fructose, cellulose, pectins, and sucrose; proteins (4-15% dry weight); amino acids (4-6.5% dry weight) like theanine, tryptophan, glycine, glutamic acid, aspartic acid, serine, leucine, valine, glutamic acid, tyrosine, lysine and threonine; trace

elements (5% dry weight) like calcium, chromium, manganese, magnesium, molybdenum, copper, iron, strontium, selenium, zinc, nickel, potassium, phosphorus, sodium, fluorine, cobalt and aluminum; sterols (stigmasterol), and bit amounts of lipids (linoleic and a-linolenic acids), pigments (chlorophyll, carotenoids), vitamins (B, C, E), xanthic bases (theophylline, caffeine) and the volatile compounds (alcohols, hydrocarbons, esters, lactones and aldehydes) (Belitz and Grosch, 1997; Dewick, 2002; Sinija and Mishra, 2008; Perry, 2009). On average, fresh leaves contain 3-4 percent of alkaloids and also phenolic acids are found in green tea like gallic acids (Graham, 1992) as given in **Table 6**.

Table 6: Chemical composition of green tea leaves

| S.No | Chemical components | Percentage of dried leaf |
|------|---------------------|--------------------------|
| 1. | Catechins | 30-40 |
| 2. | Flavonoids | 2-10 |
| 3. | Other flavonoids | 2-4 |
| 4. | Proteins | 4-15 |
| 5. | Carbohydrates | 7-25 |
| 6. | Caffeine | 0.4-15 |
| 7. | Minerals | 5-8 |
| 8. | Ascorbic acid | 1-2 |
| 9. | Gallic acid | 0-5 |
| 10. | Methylxanthenes | 7-8 |
| 11. | Amino acids | 4-6.5 |
| 12. | Volatile compounds | 0.02 |
| 13. | Alkaloids | 3-4 |

Source: Belitz and Grosch, 1997; Sinija and Mishra, 2008.

A significant amount of polyphenols such as flavanols, flavonoids, phenolic acids and flavandiols are found in the green tea infusion; these compounds might constitute around

45% of the dry mass. Flavonols, also known as catechins, are predominantly green tea polyphenols (GTPs). (Chacko et al., 2010).

2.5.3.1 Catechins

These are the green tea polyphenolic compounds which are responsible for its various health beneficial effects (Khan and Mukhtar, 2007). Catechins are the primary components of green tea and key catechins in green tea are: (-)- epigallocatechin gallate (EGCG) with a concentration (50.2 to 369.90 mg/g), (-)-epicatechin (EC) (5.78 to 133.90 mg/g), (-)-epigallocatechin (EGC) (13.44 to 288.80 mg/g), (-)-epicatechin gallate (ECG) (3.04 to 126.60 mg/g) (McKay and Blumberg, 2002; Pekal et al., 2012). The catechin structure contains polyphenolic ring (A) containing a six-membered oxygen with heterocyclic ring (C) that is attached to another polyphenolic ring (B) at the 2 position. They are thus distinguished by several hydroxyl groups on ring A and B. Epicatechin is an epimer comprising of 2 hydroxyl groups with a B ring at 3' and 4' positions and C ring with hydroxyl group at 3 positions as depicted in **Fig 12**. The only structural difference between EGC and EC is that EGC has an additional hydroxyl group at the 5' position of the B ring. Therefore, EGCG and ECG are EC and EGC ester derivatives, respectively, and are derived from gallate esterification at three C-ring hydroxyl positions (Higdon and Frei, 2003; Khan and Mukhtar, 2007).

The EGCG (Epigallocatechin- 3-gallate) is considered to be the key catechin portion of green tea with highest biological properties responsible for its major antioxidant potential that constitutes about 50 - 70% catechins in GT (Okla et al., 2017; Wang et al., 2014). While other reports demonstrated that ECG is the primary component responsible for various health benefits of green tea (Zaveri, 2001). The amount of catechin content present in green tea is influenced by many elements like topographical location, developing conditions (climate, soil, fertilizers and agricultural practice), the category of green tea (for example; decaffeinated, blended and instant), and infusion making (quantity used, temperature, boiling time) (Hakim et al., 2000; Wu and Wei, 2002). The various health beneficial properties of green tea are considered due to their catechins content **Fig. 13**.

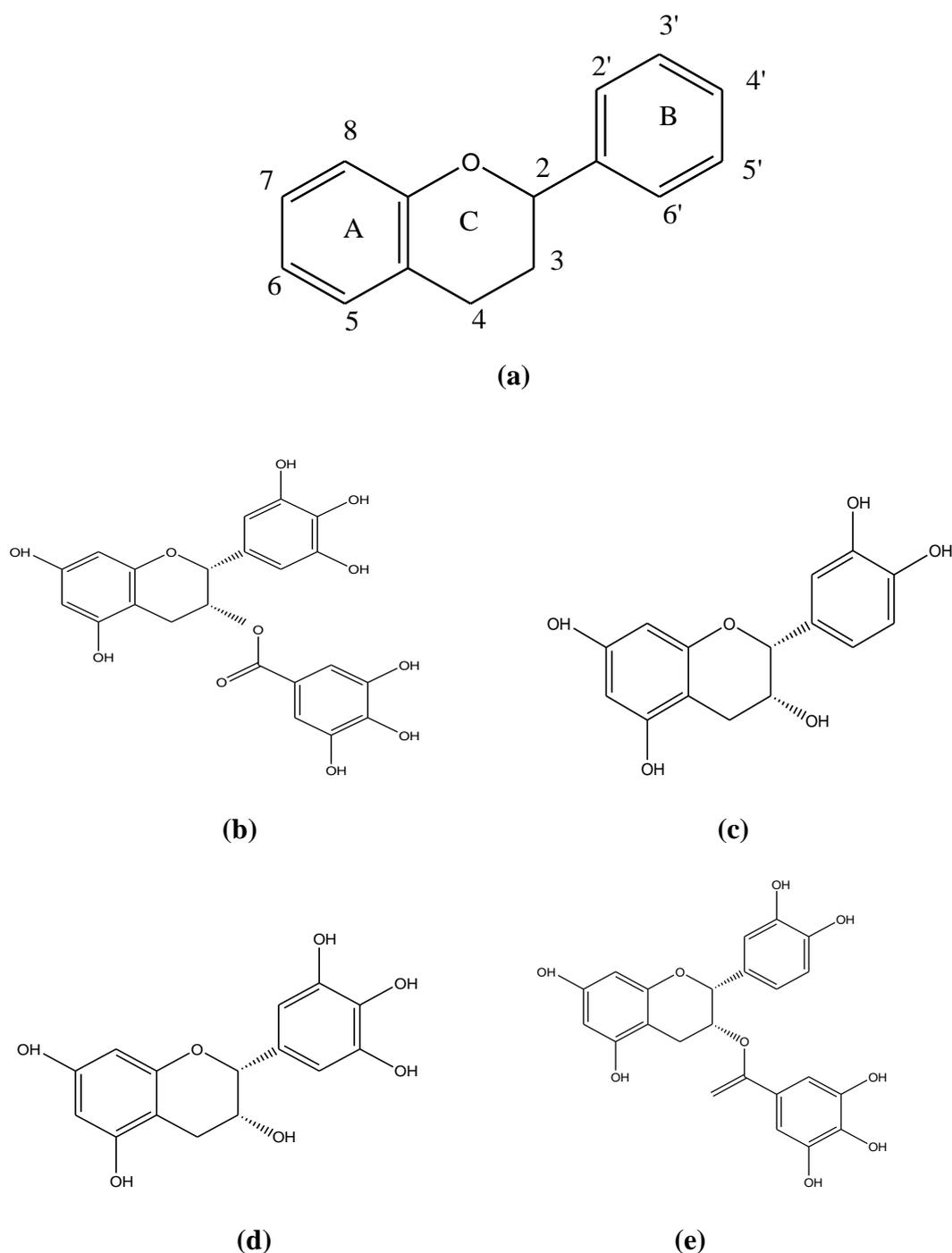


Fig. 12: Structure of major catechins of Green tea (a) basic skeleton (b) [(-)-epigallocatechin gallate]; EGCG (c) [(-)-epicatechin]; EC (d) [(-)-epigallocatechin]; EGC and (e) [(-)-epicatechin gallate]; ECG.

2.5.3.2 Taste and Flavour

Instead of above mentioned properties, green tea is having refreshing taste and flavor. The flavor and characteristic taste of green tea also comes from the different chemical compounds like polyphenols compounds, volatile terpenes, caffeine and amino acids (Yamanashi, 1995). For its strong scent, meaty, aromatic, nutty, fruity, metallic, green, potato, cucumber-like,

popcorn-like, hay-like features and the various volatile compounds of green teas are responsible (Kumazawa and Masuda, 2002). In green tea, the flavoring compounds such as hotrienol, geraniol, a-terpineol and linalool as the main flavoring constituents (Pripdeevech and Machan, 2011).



Fig. 13: Health beneficial properties of green tea

2.5.3.3 L-theanine (γ -glutamylethylamide)

It is an important amino acid present in green tea leaves that plays a vital role in tea quality and function (Cheng et al., 2019). The L-theanine chemical structure is shown in **Fig.14a**. It is believed to be responsible for flavor of green tea leaves and constitutes dry weight of 1 to 2 percent of the tea (Nobre et al., 2008). It demonstrated physiological effects such as relaxation and alertness property by enhancing the alpha brain wave activity and activation of dopamine uptake that is released in brain (Kobayashi et al., 1998; Nobre et al., 2008; Camfield et al., 2014).

2.5.3.4 Caffeine

This is a natural alkaloid found in different drinks, including tea, coffee, soft drinks, as well as products containing chocolate or chocolates (Andrews et al., 2007). The structure of caffeine is given in **Fig. 14b**. In green tea serving (230ml) about 30-50 mg of caffeine content

was found (Heckman et al., 2010). Caffeine employs various stimulatory effects on central nervous system, enhances mood and respiratory rate and causes broncho-dilatation, stimulates lipolysis, and increases diuresis (Chou and Benowitz, 1994). Caffeine intake in excess can also cause various adverse effects like headache, stomach problems, anxiety and insomnia (Jee et al., 1999). The amount of caffeine is exaggerated by numerous elements such as tea type, way of brewing and growing conditions (Lin et al., 2003). The bagged tea was discovered to have more caffeine content than loose leaves, but less than the powdered form of green tea (Matcha), this is due to higher extraction of caffeine in crushed and powdered form of infusion (Astill et al., 2001). It is observed that older tea leaves have less caffeine content than young leaves and buds (Lin et al., 2003).

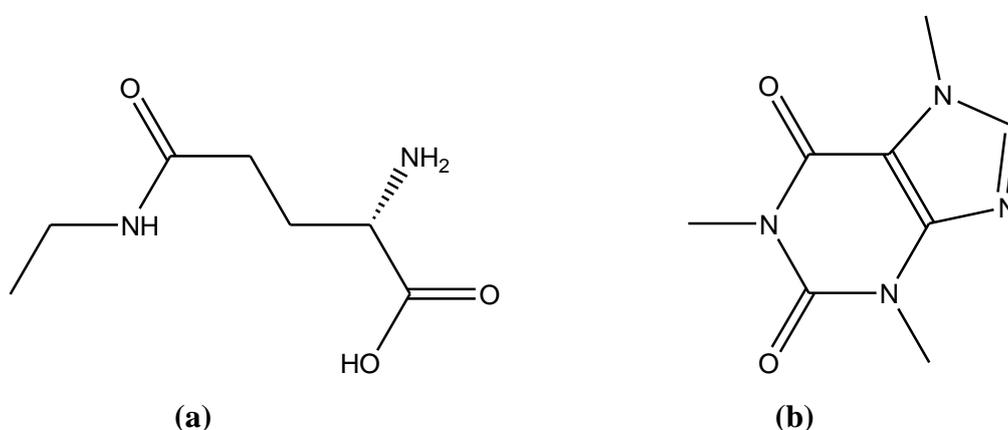


Fig. 14: Structure of (a) L- theanine and (b) Caffeine

2.5.4 Cancer chemopreventive action of green tea

The excessive generation of ROS degrades the biomolecules for example carbohydrates, proteins, lipids and nucleic acids that cause oxidative stress (Tong et al., 2015). The antioxidants are substances that are known as free radical scavengers, thus increase the cellular defense by nullifying the oxidative damage (Pal and Nimse, 2006). Two classes of antioxidants, enzymatic and non-enzymatic are available that balance the production of ROS, and our body uses enzymatic defense system to protect it from ROS (Koruk et al., 2004). Enzymatic antioxidants play their role by either removing or breaking down free radicals. The enzymatic antioxidants produced in our body are glutathione peroxide (GSHPx), glutathione reductase (GR), catalase (CAT), superoxide dismutase (SOD) and peroxiredoxin (Shahidi and Zhong, 2010). Non-enzymatic antioxidants however interfere with free radical chain reactions and are of two types: natural and synthetic antioxidants (Nimse and Pal,

2015). The natural antioxidants are present in beverages, food, fruits, vegetables and plant polyphenols (Shahidi and Zhong, 2010). However the synthetic antioxidants are compounds to which food items are added in order to continue product shelf life, mainly by preventing the oxidation of fatty acids. The two common examples of synthetic antioxidants used today are butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) (Shebis et al., 2013). Green tea's antioxidant potential is due to aromatic polyphenolic compounds that scavenge lipid-free radicals through hydroxyl groups (Senanayake, 2013). Previous reports revealed that catechins, the major polyphenolic compounds of green tea are electron donors that scavenge the ROS (superoxide anions, singlet oxygen and peroxy radicals) (Guo et al., 1999; Michalak, 2006; Nakagawa and Yokozawa, 2002). Another mechanism by which catechins demonstrate antioxidant potential is by manipulating the effective redox transition-metal ions. The polyphenolic compounds consist of hydroxyl and carboxyl groups capable of binding to copper and iron in particular (Michalak, 2006), such ions are capable of inducing free oxidation of radicals by disintegrating the lipid hydroperoxides via the hemolytic splitting of O–O bond thus creating lipid alkoxyl radicals. The lipid peroxidation was reduced using green tea catechins by simply binding the lipid alkoxyl radicals and that action is determined by the structure, number and location of hydroxyl group in these compounds (Milic et al., 1998). These catechins also revealed antioxidant potential via induction of antioxidant enzymes and inhibition of pro-oxidant enzymes (Velayutham et al., 2008). Due to its sufficient concentration in green tea and the presence of the galloyl group on the B and D ring, the major component of green tea (EGCG) is often studied (Forester and Lambert, 2011). By the help of EPR (electron paramagnetic resonance) spectroscopy, it was found that when EGCG responds to O₂, oxidation of D ring takes place and also scavenge O₂⁻ and OH radicals (Shi et al., 2000; Severino et al., 2009). Earlier studies found that GTP has a protective function in rats against azathioprine (AZA)-induced liver injury via antioxidant, anti-inflammatory and anti-apoptotic mechanisms (El-Beshbishy et al., 2011). The catechins of green tea or green tea minimize the immersion and accretion of lipophilic organic compounds in tissues, thus demonstrated its nontoxic and effective lipid-lowering therapeutic effect (Koo and Noh, 2007). Green tea administration revealed a protective function against serum malondialdehyde caused by paracetamol, catalase activity and vitamin C reduction (Ojo et al., 2006). The regular consumption of GT was found to upsurge the plasma antioxidant potential and decline the lipid peroxidation (Rietveld and Wiseman, 2003). A study showed reduction in plasma peroxides and DNA damage and increase in antioxidant status by consuming 2 cups of green tea for 42 days (Erba et al., 2005).

Green tea and its components are found to scavenge the free radicals produced from the toxicants, therefore lead to reduction in DNA damage (Chen et al., 2017). A decrease in UVB irradiation due to decrease in formation of hydrogen peroxide was observed by treatment of green tea extract (Wei et al., 1999). The bleomycin induced-DNA damage in leukocytes with EGCG therapy was found to decrease (Glei et al., 2006). A report revealed that green tea consumption (four cups/day for four months) showed a significant decline (31 %) in 8-hydroxydeoxyguanosine (8-OHdG) urinary levels, in smokers group (Hakim et al., 2003). The B(a)P prompted chromosomal damage in the blood of mouse was reduced by oral intake of green and black tea for a period of 28 days (Sasaki et al., 1993). Green tea intake is related with reduction in tumor initiation power of potent carcinogen B(a)P by reducing the levels of DNA adducts (Muto et al., 1999). The consumption of GT demonstrated a 2% reduction in 2-amino-3-methylimidazo[4,5-]quinoline-induced DNA adducts in rats (Xu et al., 1996). Similarly, the intake of green tea and encapsulated green tea infusions continually for 1-4 weeks was reported to reduce the activity of oxidative status related biomarkers (McKay and Blumberg, 2002). Clinical studies demonstrated that 40 smokers (male) in China and 27 (male and female) in United States and, both smokers and non-smokers showed a decrease in free radical generation, lipid peroxidation and DNA damage after consumption of green tea (6 cups/day) for seven days (Klaunig et al., 1999; Cabrera et al., 2006).

The exact mechanism of green tea's anticancer activity and chemoprevention is not clearly known. However, the various mechanism involved in action of green tea chemoprevention are; altering carcinogen metabolism, scavenging reactive oxygen species, enhancing the activity of antioxidant system, suppressing cell proliferation, decrease inflammation, induction of apoptosis and enhance antitumor immunity as depicted in **Fig. 15**.

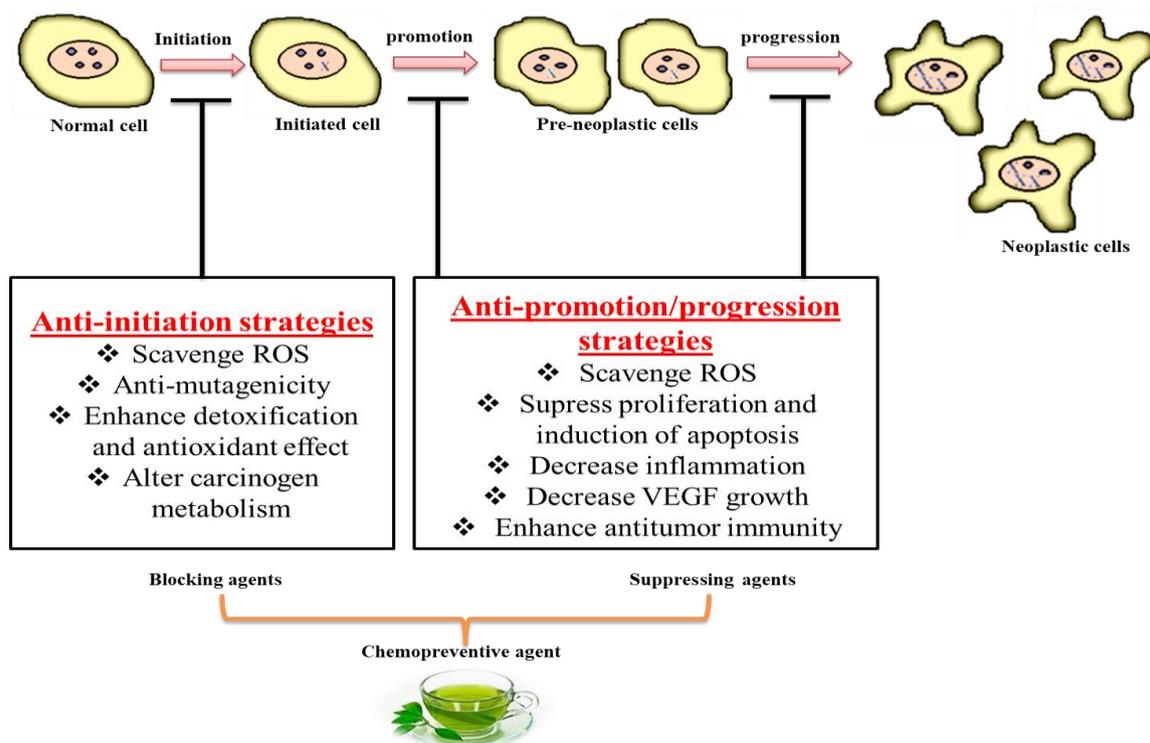


Fig. 15: Different strategies by which green tea show cancer chemopreventive property

The reduction in bio activation of carcinogen and stimulation in detoxification of enzymes are measured as one of the key mechanism involved in cancer prevention (Williams, 1984; Kamaraj et al., 2007). The naturally occurring phytochemicals (blocking agents) prevent the carcinogen activation by increasing their detoxification (Ravichandran et al., 2011). In the metabolism of carcinogens the cytochrome P450 (CYP) plays an important role (Miller and Romos, 2001). Thus modulation in their activities and expression by various plant extracts is a potential cancer preventive mechanism (Gross- Steinmayer et al., 2004). The earlier studies reported that green tea modulates the activities of phase II enzymes (epoxide hydrolase, glutathione-S-transferases/glutathione, quinone reductase and UDP glucuronosyltransferases) (Katiyar et al., 1993; Steele et al., 2000). The action of aryl hydrocarbons that induced cancer by interfering the transcriptional activation of both CYP1A1 and CYP1A2 in animals and are blocked by green tea. The green tea reduces human CYP1A promoter transcription- a guided receptor gene induced by 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) AhR ligand and inhibits accumulation of CYP 1A1 and CYP 1A2 mRNAs. Green tea and EGCG inhibits the TCDD-mediated binding of AhR to the transcription of DNA and CYP1A (Williams et al., 2000). The activities of antioxidant enzymes (Catalase and Superoxide dismutase) were found to be increased by green tea catechins through nuclear factor erythroid 2-related factor antioxidant response element (Nrf2 – ARE) signaling pathways (Na et al., 2008).

Long term inflammation causes unnecessary release of superoxide and nitric oxide (NO) anion that interacts with each other and form peroxynitrite. The polyphenols of green tea acts as a strong nitric oxide synthase gene expression inhibitors that play a vital role in the prevention of cancer (Srivastava et al., 2000). The cyclooxygenase-2 (COX-2) and nitric oxide synthase (iNOS) inducible enzymes plays a vital role in inflammation and induction of its function is associated with cell proliferation and inhibition of apoptosis. The expression of COX-2 and/or iNOS suppressed by green tea which are related to various type of cancers and inflammatory diseases by delaying the transcription factor stimulation, kappa nuclear factor-B [NF-kappa B] (Surh et al., 2001). Cox-2 plays a crucial role in the growth of lung cancer and to act a possible biomarker for tumor growth (Dannenbergh and Subbaramaiah, 2003).

The immune system plays a crucial role in body's battle against various diseases and foreign agents to prevent diseases like cancer (Little et al., 2005). Green tea improves humoral and cell-mediated immunity and reducing the risk of various types of cancers (Klein et al., 2000). Inflammation is also one of the immune response and long term inflammation may cause various diseases. The level of lipopolysaccharide induced tumor necrosis factor (TNF) was observed to be decreased by green tea polyphenols (Yang et al., 1998). PolyE and EGCG administration decreased the level of certain inflammatory cytokines such as TNF in the colorectal epithelium and blocked carcinogenesis associated with inflammation in the mouse model (Shirakami et al., 2008). All these results demonstrated that administration of green tea catechins can have effect on inflammatory conditions that are prevented by anti-inflammatory behavior and activation of NF- κ B is prevented (Butt and Sultan, 2009).

For cell survival and proliferation, apoptosis (programmed cell death), which involves a variety of complex pathways and enzymes, plays an ideal function. The proliferation of abandoned cells due to the absence of programmed cell death is closely linked to carcinogenesis (Gusman et al., 2001). Green tea was investigated in both cancer cell lines and lung tissues of mice to serve as a possible inducer of cell apoptosis (Yang et al., 2000; Gupta et al., 2001). EGCG promotes UV-induced apoptosis and cell survival through phosphorylation of Bad protein (Ser 112 and Ser 136) through Akt and Erk pathways by enhancing the Bcl-2 to Bax ratio (Chung et al., 2003). Green tea and its active component (EGCG) inhibit the activation of the AP-1 transcription factor (a role in apoptosis) that may be due to inhibition of pathway-dependent jun NH2 terminal kinase (JNK) (Chen et al., 1999). It is found that green tea polyphenols cause programmed death of cells in colon, brain, cervical, prostate, blood and liver cancer cells (Gupta et al., 2003; Hastak et al., 2003; Ahn et al., 2003; Chen et al., 2003). Administration of GT (0.6%) caused cell apoptosis in

lung adenomas (Liao et al., 2004). The catechins of GT prompted cell apoptosis that is related to up-regulation of p53 and p21 apoptosis protein expression (Zou et al., 2010).

The new production of blood vessels is important for tumor growth and metastasis, known as angiogenesis (Kerbel, 2000). Angiogenesis is the biomarker and target for cancer chemoprevention (Folkman, 2003). Tumor cells are not capable to grow beyond 1-2mm³ in the absence of angiogenesis and will die due to lack of oxygen supply (hypoxia) (Folkman, 1990). During hypoxia, the live cells in the surrounding environment are able to produce the specific growth factors that stimulate the cell proliferation and increased the cell division (Nishida et al., 2006). Some of the growth factors (TGF- α ; transforming growth factor, bFGF; basic fibroblast growth factor, TNF- α ; alpha-tumor necrosis factor and VEGF; vascular endothelial growth factor) (Distler et al., 2003). The CAM (chick embryo chorioallantoic membrane) assay, an *in vivo* model is used to test the anti-angiogenic properties of various components (Ribatti et al., 2001). The instillation of 0.6% of green tea demonstrated an inhibition in angiogenesis and VEGF expression in lung samples of mice (Liao et al., 2004). The GT components are found to decrease the angiogenesis on CAM that was induced by angiogenin like protein (Maiti et al., 2003). By blocking VEGF activation and VEGF dependent tyrosine phosphorylation of VEGFR-2, EGCG inhibits angiogenesis, thus preventing VEGF-dependent angiogenesis (Jung et al., 2001; Lamy et al., 2002). Green tea infusion also prevents HUVECs (human umbilical vein endothelial cells) angiogenesis by suppressing VEGF receptor expression (VEGFR-1 and VEGFR-2), suggesting that by inhibiting VEGF receptors, is one of the mechanisms by which green tea polyphenols employ their anti-angiogenic effect (Kojima-Yuasa et al., 2003). The *in vivo* investigations proved that EGCG plays a key role in angiogenesis inhibition, thus proposing a possible correlation between consumption of tea and angiogenesis dependent diseases prevention and treatment including cancer (Cao and Cao, 1999)

2.5.4.1 Animal and clinical studies

In various animal models green tea and its constituents demonstrated a reduction in carcinogens such as benzo[a]pyrene, N-nitrosodimethylamine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) induced lung tumours in an A/J mouse model (Liao et al., 2004; Clark and You, 2006). The oral installation of GT infusion in mice illustrated a decline in number of lung carcinoma thus suggesting green tea a preventive agent against lung tumorigenesis (Yang et al., 2002). The N-nitrosodiethylamine (NDEA)- induced lung tumor occurrence and tumor volume was found to be decreased in A/J mouse that was treated with decaffeinated green tea and green tea (Wang et al., 1992). In both p53 mutant and wild-type

mice, green tea decreased the NNK caused lung tumorigenesis (Zhang et al., 2000). The urethane-induced lung neoplasia suppressed by the treatment of green and oolong tea in Kummung mice (Wu et al., 1987). In A/J mice, a protective effect of aqueous infusion of green tea against B(a)P and NDEA mediated lung and forestomach tumorigenesis was observed (Wang et al., 1992). The green tea demonstrated a preventive effect at all stages of carcinogenesis by inhibiting the growth and reduction in recognized benign tumors (Wang et al., 1992). The NNK induced lung tumor was found to be reduced in mice by the oral treatment of polyphenon E and caffeine for 32 weeks (Lu et al., 2006). The intestinal tumor formation was observed to be decreased by infusion of GT alone and its combination with sulindac in ApcMin/+ mouse (Suganuma et al., 2001). Oral intake of green tea daily over a span of 10 years slowed the cancer onset in both smokers and non-smokers (Nakachi et al., 2000). Epidemiological evidences showed a decreased risk of lung cancer associated with high green and black tea intake, especially in those who are never smokers (Arts, 2008). A clinical study (n = 472) reported that patients of breast cancer at stage I and stage II had a incidence of recurrence (16.7%) alone and a disease-free period of 3.6 years after drinking more than 5 cups of green tea compared to less than 4 cups a day (Nakachi et al., 1998). Increased intake of green tea (2 cups per day) has been reported to be associated with an 18% decrease in lung cancer incidence (Tang et al., 2009). In women who drink green tea (90ml/day) everyday, a substantial reduction in breast cancer has been observed compared to those who take tea once a month (Wu et al., 2003).

2.6 Meta-Analysis

A systematic method (meta-analysis) is based on synthesizing the data from multiple scientific studies rather than results of individual studies. That is why it is considered more exact and reliable approach than other qualitative approaches for reviewing the research (Cook and Leviton, 1980). Various studies of meta-analysis revealed that GT intake is correlated with mild to moderate effects on four main worldwide assassins i.e. type II diabetes, cancer, stroke, and atherosclerosis related cardiovascular events (Wu et al., 2003; Ogunleye et al., 2010; Johnson et al., 2012; Wang et al., 2012). One meta-analysis report included 22 studies proved that an increase in intake of green tea (2 cups per day) is associated with 18% reduction in development of lung cancer and high green tea may be correlated with decrease in incidence of lung cancer (Tang et al., 2009). One more report done on 38 lung cancer studies in which (26 case-control studies and 12 cohort studies) demonstrated that drinking green tea is correlated to decreased lung cancer in female but no association in male or cohort studies has been observed (Wang et al., 2012). In this report 20

studies were undertaken for observation, out of which 4 studies demonstrated significantly reduced risk of lung cancer by increased consumption of green tea. In non-smoker's intake of tea was found to be significantly protective against lung cancer (Arts, 2008). From the data of 9 studies it was found that consuming more than 3 cups of green or black tea/day demonstrated 21% lesser risk of stroke than the individuals that consume less than 1 cup of tea per day. This report suggests that taking 3 cups of tea daily can prevent the ischemic stroke (Arab et al., 2009). Another report revealed that intake of green tea displayed a reduction in total cholesterol, LDL cholesterol and systolic blood pressure and systolic blood pressure was low as compared to total and LDL cholesterol level that was moderate (Onakpoya et al., 2014).

To improve the health promoting effects of green tea, one of the approaches is to combine it with medicinal plants which are used as herbal teas or tisanes. Tisanes are prepared in the same way as that of tea but are not tea at all because they do not belong to plant *Camellia sinensis* from which all teas are made (Killedar and Pawar, 2017). The growing awareness of health about the role of diet in an individual's general well-being was the main reason for preference choice for most herbal teas (Joubert et al., 2017). Herbal teas are produced from different parts of plants like fruits, flowers, seeds, leaves, roots and stems of other than *C. sinensis*, used since thousands of years for nutrition and diseases prevention (Deetae et al., 2012). They are simple and effective ways to take advantage of aroma and health benefits from herbs (Killedar and Pawar, 2017). It was reported that there is link between the herbal tea consumption and reduction in various ailments (Horzic et al., 2009). There may be synergistic, additive or antagonistic interactions in the mixture of different plant-based foods that may affect their biological properties (Gawlik-Dziki, 2012). The antioxidant synergistic interactions between teas and herbal infusions will improve the chemopreventive capacity of redox imbalance related diseases (Phan et al., 2018). A study showed antagonistic, synergistic or additive antioxidant interactions by augmenting its own catechins and flavonols with whole green tea extract (Colon and Nerin, 2016). In various antioxidant assays, additive, synergistic or antagonistic interactions were revealed by the ternary combination of green tea, rosemary leaves (*Rosmarinus officinalis*) and Persian oak fruit (*Quercus brantii*) at 1:1:1 ratio (Nedamani et al., 2015). A synergistic radical quenching ability was demonstrated by a polyherbal combination of gooseberry (*Phyllanthus emblica*), ginkgo (*Ginkgo biloba*), grapes (*Vitis vinifera*), pomegranate (*Punica granatum*), cinnamon (*Cinnamomum cassia*), and green tea (*Camellia sinensis*) (Jain et al., 2011). Much of the earlier research on the relationship of

green tea or its phytochemicals with other plants or their active components used various solvent systems to prepare green tea combinations (Liu et al., 2016; Pan et al., 2017).

The remedial plants examined in this report are *Ocimum gratissimum* (Vana Tulsi), *Cymbopogon flexuosus* (East Indian lemongrass), *Cymbopogon citratus* (West Indian lemongrass) and *Hibiscus rosa – sinensis* (China rose), which are reported to be rich in antioxidants and are used as herbal teas (Naithani et al., 2006; Jadhav et al., 2009).

2.7 Medicinal plants used in this study

2.7.1 *Ocimum gratissimum*

The perennial plant *O. gratissimum* (OG) that belongs to the family Lammiaceae is generally famous by the name of “vana tulsi” in India (Monga et al., 2017). This plant is native to tropical areas specifically India and West Africa (Prabhu et al., 2009). It is also known as scent leaf and is used as a flavor enhancing agent in foods (Dubey et al., 2000; Ezekwesili et al., 2004). The taxonomic status of *O. gratissimum* is given below.

Table 7: Classification of *O. gratissimum* plant

| | |
|-----------------|--------------------|
| Kingdom | Plantae |
| Division | Tracheophyta |
| Class | Magnoliopsida |
| Order | Lamiales |
| Family | Lamiaceae |
| Genus | <i>Ocimum</i> |
| Species | <i>gratissimum</i> |

2.7.1.1 Morphological features

O. gratissimum is a herbaceous plant upto height (1.9 m) with branched stems. Its possess opposite leaves about 10cm long and 5cm wide, with ovate to ovate-lanceolate, sub-acuminate to apex acuminate, cuneate and base decurrent with a coarsely crenate on both sides, pubescent and spotted. Presence of glandular trichomes is also observed on leaves. The petioles are about 2-4.5 cm long, slender and pubescent. The peduncles are heavily pubertal. The calyx (5mm long), campanulate (5-7 mm long) and in color it is greenish-white to greenish-yellow (Bhat, 2003).



Ocimum gratissimum

2.7.1.2 Benefits

The *O.gratissimum* extract prepared by different methods (maceration, decoction, or infusion) and solvents (water, ethanol or methanol) were used in treatment of various ailments of inflammation (Erinoso et al., 2012). It possesses ample medicinal properties like antioxidant activities, vasorelaxation, wound healing, anti-inflammatory, immunostimulatory, anthelmintic, antibacterial, antifungal, and antiviral activities, whereas recent research also efforts on cancer chemoprevention and immunomodulation (Costa et al., 2012; Mahapatra et al., 2011; Nangia-Makker et al., 2007). All the health benefits listed above may be due to phytochemicals (flavonoids and polyphenols) and volatile compounds (eugenol, thymol, geraniol and also contains xanthenes, terpenes and lactones) present in it (Dubey et al., 2000; Ezekwesili et al., 2004).

2.7.2 *Cymbopogon citratus* (CC)

Cymbopogon citratus known as West Indian Lemongrass is an aromatic herb belongs to family Poaceae. This herb is native of India, tropical Asia and is nowadays distributed throughout the world (Costa et al., 2012). This herb is of high economic value due to use in various pharmaceutical, cosmetic and in industries of food (Oyedele et al., 2002; Saddiq and Khayyat, 2010; Coradi et al., 2014). The botanical classification of this plant is given below.

Table 8: Classification of *C. citratus* plant

| | |
|-----------------|-------------------|
| Kingdom | Plantae |
| Division | Tracheophyta |
| Class | Magnoliopsida |
| Order | Poales |
| Family | Poaceae |
| Genus | <i>Cymbopogon</i> |
| Species | <i>citratus</i> |

2.7.2.1 Morphological features

The leaf margins of *C. citratus* plant are entire. The shape of the leaf is linear with a size of 5-7cm long. The venation of the leaf is parallel small components can be seen as small sticks and leaves are glabrous in parallel. The lamina tip and base are acuminate and sheath and color of the leaf is pale green (Yesil and Akaln, 2015).



Cymbopogon citratus

2.7.2.2 Benefits

It possesses various medicinal properties such as antioxidant, antiseptic, antispasmodic, anticonvulsant, analgesic, hepatoprotective, antirheumatic, antimicrobial, antifungal, antimutagenic, antiinflammatory, antimalarial, antihepatotoxic, gastrointestinal ailments and used against nervous system diseases (Shah et al., 2011; Koh et al., 2012; Manvitha and Bidya, 2014). Previous reports showed that the aerial parts of *C. citratus* contain triterpenes, polyphenols and essential oils (Cheel et al., 2005; Figueirinha et al., 2010). The main ingredient in its essential oil is citral with a strong lemon fragrance and the above activities may be related to citral, and is considered as a potent scavenger of ROS (Halabi and Sheikh, 2014). The various factors that alter the chemical composition of essential oils in *C. citratus* are as; terpenes, esters, ketones, alcohols, mainly aldehydes and geographical origin (Manvitha and Bidya, 2014).

2.7.3 *Cymbopogon flexuosus* (CF)

C. flexuosus is one of the most essential cultivated aromatic plants in tropical and subtropical areas of India, Madagascar, Indonesia and in countries of Africa and South America (Mandal and Bhattacharya, 2015). In India *C. Flexuosus* grows mainly in Kerala, Tamil Nadu and it belongs to the family of Poaceae (Dhinesh et al., 2016). In India *C. flexuosus* commonly known as East Indian lemongrass and its other important species that is used in treatment of issues with arthritis, cellulites, skin-related issues and serve as digestive tonic (Oussalah et al., 2006; Wahab et al., 2018). The botanical classification of this plant is given below.

Table 9: Classification of *C. flexuosus* plant

| | |
|-----------------|-------------------|
| Kingdom | Plantae |
| Division | Tracheophyta |
| Class | Magnoliopsida |
| Order | Poales |
| Family | Poaceae |
| Genus | <i>Cymbopogon</i> |
| Species | <i>flexuosus</i> |

2.7.3.1 Morphological features

The *C. flexuosus* is a tall plant having short and thick rhizome, the culm grows up to 3m tall with a nodding inflorescence. The stem is erect, smooth, polished, solid and globous at nodes. The leaf blades are linear acuminate tapering at both ends. The leaf is hairy on the upper surface and shortly hairy on the lower surface near the junction with sheath over 1, on length and 1.5cm on width (Marigowda et al., 2016).



Cymbopogon flexuosus

2.7.3.2 Benefits

The essential oil present in *C. flexuosus* leaves gives scientific evidence that supporting its immunostimulatory, anti-inflammatory, antimicrobial, antioxidant and antifungal properties by using different models (Tsai et al., 2011; Adukwu et al., 2016). *C. flexuosus* leaves are used in antiseptic, cosmetics and for treatment of fever (Desai and Parikh, 2012). It also possesses chemopreventive potential against Ehrlich and Sarcoma-180 tumors (Sharma et al., 2009).

2.7.4 Hibiscus rosa - sinensis (HR)

H. rosa – sinensis, a flowering plant belongs to family Malvaceae (Purushothaman et al., 2013). It is a smooth bush that is native of China and widely cultivated in gardens as an ornamental herb throughout India with different color of flowers (Jadhav et al., 2009). It is

commonly known as China rose (Al-snafi, 2018). It grows best under moderate temperature and relatively high humid conditions (Sharma et al., 2001). The botanical classification of this plant is given below.

Table 10: Classification of *H. rosa-sinensis* plant

| | |
|-----------------|----------------------|
| Kingdom | Plantae |
| Division | Tracheophyta |
| Class | Magnoliopsida |
| Order | Malvales |
| Family | Malvaceae |
| Genus | <i>Hibiscus</i> |
| Species | <i>rosa-sinensis</i> |

2.7.4.1 Morphological features

The plant of *H. rosa-sinensis* grows about 1.5–3 m wide and 2.5–5 m tall, with shiny leaves, bright color flowers in summer and autumn (Rassem et al., 2017). The flowers of this plant are ebracteate, complete, regular, bisexual and protandrous hypogynous. The epicalyx is 5, free and linear. The calyx is also 5, gamosepalous, inferior and green. Corolla 5, polypetalous, obovate and red in color. The androecium is many, gynoecium pentacarpellary, stigma 5 are velvety red (Gupta et al., 2005).



Hibiscus rosa-sinensis

2.7.4.2 Benefits

It is usually consumed in teas made from its leaves, flowers and roots (Da-Costa-Rocha et al., 2014). It exhibited many biological properties (antifertility, antipyretic, analgesic, anticonvulsive, antiestrogenic, anti-inflammatory, antispasmodic, antifungal, antiviral, CNS depressant, hypoglycemic, hair growth) and many more (Jadhav et al., 2009; Shewale et al., 2012; Khalid et al., 2014; Nayak et al., 2015).

2.8 Antioxidant interactions

The mixture of different phytochemicals gives the effect that may be greater or lesser than the sum of their individual constituent and the effects are categorized as synergism, antagonism or additive effect (Efferth and Koch, 2011). The combined effect is greater or lower than their individual effect in synergistic or antagonistic interactions and equal in additive effect (Chou, 2006). The use of combination of various dietary compounds provides one of the important advantages, the possibility of synergistic antioxidant interactions. Synergism is important because it enables the use of lower and safer doses of each compound (Boik, 2001). Whole food consumption for example vegetables, fruits and grains is strongly related to reduced cancer risk (Jensen et al., 2004; Vainio and Weiderpass, 2006).

2.8.1 Effect of interaction on biological properties

Isolated pure compounds may or may not behave in the same way as the original food component, losing their biological activity. The same health profits are not generated from single dietary phytochemicals as diet rich in vegetables, fruits and whole grains (Liu, 2004). Two or more mixtures interact with each other and affect the antioxidant, anti-lipid peroxidation, anti-inflammatory and anti-carcinogenic activities. Synergistic antioxidant interactions between kaempferol and cyanidine-3-glucoside, kaempferol and delphinidine-3-glucoside, cyanidine-3-glucoside and myricetin-3-glucoside have been observed (Hidalgo et al., 2010). Carotenoid mixtures are found to be more effective against oxidative imbalance inhibition than individual compounds (Shixian et al., 2005; Han et al., 2012). The combinations lycopene b-carotene, of b-carotene-lutein, lycopene-lutein and tocopherol-lycopene displayed stronger antioxidant potential than the individual compound, signifying potential synergy (Zanfini et al., 2010; Stinco et al., 2016). The α -tocopherol-caffeic acid, quercetin-ferulic acid combination demonstrated higher anti-lipid peroxidation activity than the individual compounds in soybean phosphatidylcholine liposome model (Becker et al., 2007; Neunert et al., 2015). In gastric carcinoma cell lines green tea polyphenols demonstrated a synergistic effect on the development of cells and apoptosis induction (Horie et al., 2005). All the cell lines differ in susceptibility when treated with EGCG but EC does

not trigger cell growth or apoptosis. Moreover, when EC was combined with other catechin, a well-defined synergistic effect was observed on the induction of apoptosis. After treatment, caspases 3, -8 and -9 were observed and was found that these caspases in the apoptosis triggered by catechins. The investigations of using different cancer chemopreventive agents in combination with various action pathways can be a valid methodology to maximize efficiency and reduce the toxicities (Hong and Sporn, 1992; Sporn, 1980). The treatment by a single compound like polyphenon E or atorvastatin were not active in reducing lung carcinogenesis, while as the combined treatment of these two compounds significantly illustrated a reduction in tumor number and tumor load (Lu et al., 2008). The binary combinations of phytochemicals also demonstrate reduction in antioxidant potential for example the combination of alpha-tocopherol with ferulic acid or caffeic acid showed synergism against lipid peroxidation, but antagonism was shown by the combination with chlorogenic acid, which may be due to the steric structure of chlorogenic acid that makes it unable to attach with alpha-tocopherol (Neunert et al., 2015). The combination of β -carotene with flavonoids baicalein, daidzein, or polyphenols of green tea (ECG, EGCG, EC and EGC) significantly decreased the antioxidant potential (Song et al., 2011)

It is recommended to consume the whole vegetables and fruits instead of single bioactive compound (Rodriguez-Casado, 2016). The combination of various phytochemicals can produce antioxidative and non-additive effects. The combination of green tea phytochemicals such as GCG, gallic acid, gallic acid gallate; EGCG, epigallocatechin gallate; CG, catechin gallate and ECG, epicatechin gallate demonstrated synergistic effect (Colon and Nerin, 2016). The hyperoside + EGC binary combination showed significant synergistic effects at 3:1 ratio. The synergistic interaction between EGC and hyperoside can therefore be considered the one explanation for synergistic effects of antioxidant in PFE + GTP combination (Liu et al., 2016). Binary combination of green tea with plants such as *Punica granatum*, *Phyllanthus emblica* L., *Vitis vinifera*, *Cinnamomum cassia*, *Ginkgobiloba* L. increases the antioxidant potential synergistically and also lower the concentration of each herb used (Jain et al., 2011). The EGCG combination with curcumin demonstrated the synergistic effect by reducing cancer cell growth by blocking the cell cycle (Khafif et al., 1998; Balasubramanian and Eckert, 2004). It was discovered that combination of green tea polyphenol, vitamin C and vitamin E could work synergistically to defend lipid peroxidation, and antioxidant synergy via regeneration of vitamin C and vitamin E by GT polyphenols (Dai et al., 2008). In human breast cancer cells (MDA-MB-231 cells) EGCG with 4-methylumbelliferone, demonstrated synergistic interaction, but with hydroxytyrosol (HT) and TNP-470 showed additive

interactions, and a strong antagonism with cholecalciferol (Vit D3) followed by placlitaxel, DMF, kahweol, sunitinib, leflunomide and metformin (Garcia-Vilas et al., 2016). The infusion of tea, ginger, black pepper and tulsi (1:1:1:1) and tea, ginger and tulsi (1:1:1) demonstrated excellent antioxidant action compared to other extracts and mixtures (Gupta et al., 2014). The mixture of black or green tea with phytochemicals from soy, synergistically suppressed prostate (human) and breast (mice) tumor growth (Zhou et al., 2003; Zhou et al., 2004). The quercetin and EGCG combination displayed an increased inhibitory effect on lung cancer cell proliferation (Wang et al., 2012). While numerous studies indicate that complementary mechanisms of individual components can contribute to increase the cancer chemopreventive potential when used in combination, care is needed as certain combinations can display antagonistic effect which diminished the effects and may even serve as a promoter of carcinogenesis in some cases (Durak et al., 2014; Durak et al., 2015; Gawlik-Dziki et al., 2016).

The anti-oxidative synergy involves different mechanisms such as regeneration, sacrificial oxidation, spatial distribution and mutual protection. In regeneration, oxidation takes place in one antioxidant that become free radical and receives the hydrogen atoms from donor antioxidant to regenerate itself, weaker antioxidant regenerate the stronger one in a binary mixture of antioxidant (Becker et al., 2007; Vijayalakshmi et al., 2014). In some structures, alpha-tocopherol antioxidant regeneration maintains the radical scavenging mechanism (Becker et al., 2004). Binary antioxidant mixtures such as alpha-tocopherol and lycopene; alpha-carotene and lycopene; lycopene and rosmarinic acid; synergy-showing lycopene and glabridin, in which less active antioxidants are regenerated by a more active compound. Synergistic effects have been demonstrated through cooperative interactions between individual compounds in which the partner can be regenerated as an electron donor (Hidalgo et al., 2010). Alpha-tocopherol is regenerated by quercetin in a ternary mixture of α -tocopherol, quercetin and ascorbic acid, which is regenerated by ascorbic acid afterwards (Murakami et al., 2003). In sacrificial oxidation one antioxidant comes in contact with the singlet oxygen and prevents the oxidation of partner (Neunert et al., 2015). In a binary combination, one antioxidant can protect another via sacrificial oxidation mechanism, for example, all-trans β - carotene were protected using lycopene against isomerization caused by free radicals and the β - carotene isomerization is delayed and initiated once the lycopene is fully exhausted (Heymann et al., 2015). Lycopene's ability to protect the isomerization of β -carotene stems from its greater capacity for antioxidants (Clinton, 1998; Wang, 2012).

Initially isomerized and degraded in the reaction, lycopene acts as a quenching buffer, thereby preventing isomerization of the corresponding carotenoids (Namitha and Negi, 2010). The spatial distribution in which the two phytochemicals or antioxidants have different direction at the lipid/water interface is another mechanism which increases the interaction synergistically (Fuhrman et al., 2000; Becker et al., 2004). The liposome oxidation by binary mixture of antioxidant (lycopene and astaxanthin) was initiated by AMVN (2,2' -azobis(2,4-dimethylvaleronitrile) which was evaluated by the formation of conjugated dienes, the astaxanthin (ASTA) anchored to the lycopene and β -carotene interface persisted in the centre due to unique spatial distribution in the liposome (Liang et al., 2009). The mixture of lycopene and glabridin reduced the AAPH, 2,2' -azobis(2-amidinopropane) dihydrochloride; prompted oxidation of LDL in humans, the collaboration of radical scavenging enabled the spatial distribution of the LDL compounds (Fuhrman et al., 2000). The another mechanism is the mutual protection in which the antioxidant in a mixture behaves in various forms of anti-oxidation, protecting each other from being oxidized (Becker et al ., 2007).

In the current study, the binary combinations of green tea with medicinal plants are investigated for their possible synergistic antioxidant interactions, keeping in mind the green tea combinations available in market and also the method by which these infusions are usually prepared before ingestion.

CHAPTER 3
HYPOTHESIS

HYPOTHESIS

In the world one of the main causes of morbidity and mortality is cancer (Bray et al., 2012). Among various forms of cancers, globally leading cause of cancer deaths is lung cancer (Bray et al., 2018). It has proved to be challenging to control with surgical and therapeutic methodologies (Paci et al., 2017). The standard therapies or their combination stay unsuccessful to make a major impact on survival of patients due lack of reliable biomarkers for early stage diagnosis and to adverse side effects. These therapies are found to be less effective for lung cancer because recovery of the patients is limited (Hady, 2012). Surgery, the other option for lung cancer treatment is difficult in some parts and is not valid for sub-clinical metastases as there is recurrence of this disease (Ahn et al., 2016). To turn down the mortality rate caused by lung cancer, there is an immediate need to find certain agents for cancer prevention and treatment, with less or no toxic effect. One of the approaches is chemoprevention through use of natural or artificial substances that can prevent or halt the progression of cancer development (Chikara et al., 2018). The various plant infusions or their phytochemicals have shown promising cancer chemopreventive outcomes (Fernandez et al., 2006; Na et al., 2008; Cao et al., 2016). Since ancient times beverages such as teas (obtained from plant *Camellia sinensis*) and herbal teas (other than *Camellia sinensis*), are being utilized to impart better health and for treatment of various disorders like headache, inflammation and cancer (Heck and de-Mejia, 2009). There are various types of teas like oolong, black, green, and white tea among which commonly consumed drink in the world is green tea after black tea (Namita et al., 2012). It is becoming popular due to its various health beneficial properties such as antioxidant, anticarcinogenic, antiangiogenic, antimutagenic, antihypertensive and anti-obesity (Cabrera et al., 2006). Various meta-analysis reports revealed that green tea has mild to moderate effect on the four major global killers i.e., cancer, type II diabetes, stroke, and atherosclerosis related cardiovascular events (Tang et al., 2009; Johnson et al., 2012; Wang et al., 2012). To further enhance the efficacy of green tea, one of the methods is to mix it with other medicinal plants. The different combinations are available in the market claiming better ability of green tea combination (GTC) to prevent diseases, but a limited scientific data is available on such combinations. When two or more substances are mixed together, they may interact in synergistic, additive or antagonistic manner (Gawlik- Dziki, 2012). In the present study, green tea was combined with four medicinal plants separately such as [*Ocimum gratissimum* (Vana Tulsi), *Cymbopogon flexuosus* (East Indian lemongrass), *Cymbopogon citratus* (West Indian lemongrass) and

Hibiscus rosa-sinensis (China rose)], also used as herbal teas or tisenes. In order to explore the possible synergistic interactions the green tea combinations will be evaluated for antioxidant, antilipidperoxidation, antihemolysis and antiangiogenesis. The binary combination showing maximum efficacy will be selected for further evaluation at five different ratios (3:1, 2:1, 1: 1, 1:2 and 1:3) for their antioxidant potential. The best ratio of binary combination will be further evaluated for anticancer property by employing in vitro by determining the cytotoxicity against A549 lung cancer cell line and *in vivo* B(a)P induced lung tumorigenesis in mice model.



This investigation will provide scientific evidence to the general public about the efficacy of green tea combination to prevent lung cancer.

CHAPTER 4
AIMS AND OBJECTIVES

AIMS AND OBJECTIVES

4.1 Aims

Green tea a well-known beverage has gained popularity worldwide due to its health benefits. Many meta-analysis reports revealed that green tea (GT) has a potential to prevent cancer but to a limited extent. To further enhance the efficacy of green tea, it was combined with different herbs. To achieve the aims of this study, the following objectives were formulated as given below.

4.2 Objectives

1. Preliminary screening of antioxidant potential of green tea (GT) and green tea combinations (GTCs) and quantification of their total phenolic and flavonoid content.
2. Ex-vivo evaluation of anti-lipidperoxidation and anti-hemolysis activity of GT and GTCs.
3. In-vivo modulation of angiogenesis employing chick chorioallantoic membrane by GT and GTCs.
4. Assessment of cancer chemopreventive potential of GT and GTC against benzo(a)pyrene [B(a)P] mediated lung carcinogenesis in mice model.

CHAPTER 5
MATERIALS AND METHODS

MATERIALS AND METHODS

The plant materials selected for the present study are green tea, Vana tulsi, West Indian lemongrass, East Indian lemongrass and China rose. The infusions were screened for their antioxidant, anti-lipid peroxidation, anti-hemolytic and anti-angiogenic activities. The most effective combination was further used for *in-vivo* study.

5.1 Chemicals and reagents

The analytical grade of all the chemicals used in this analysis are: Benzo(a)pyrene [B(a)P] and vascular endothelial growth factor (VEGF) were procured from Sigma-Aldrich Chemicals, Mumbai, India. Corn-oil from deve herbes, New Delhi, 2,2-diphenyl-1-picrylhydrazyl (DPPH), the 2,2' azino-bis(3-ethylbenzthiozoline-6-sulfonic acid) (ABTS), methanol, ethanol, sodium nitroprusside, sodium nitrite (NaNO₂), O-phosphoric acid, sulphanilamide, naphthylethylene diamine dihydrochloride (NEDA), potassium persulphate, ascorbic acid, ferrous sulphate, ethylene diamine tetra acetic acid (EDTA), thiobarbituric acid (TBA), trichloro acetic acid (TCA), hydrochloric acid (HCl), Follin-Ciocalteau reagent, aluminium chloride (AlCl₃), gallic acid, quercetin, butylated hydroxytoluene (BHT), hydrogen peroxide (H₂O₂), sodium chloride (NaCl), ethylenediaminetetraacetic acid (EDTA), Tris-HCl, sodium hydroxide (NaOH), and sodium carbonate (Na₂CO₃) were procured from either S.D. Fine Chemicals, SRL or HiMedia India Ltd.

5.2 Study Materials

The green tea (Darjeeling green classic tea, Batch No. 6011, 8041 and 8062) was procured from Bud White Teas Pvt. Ltd., Delhi, India, in the form of 50 and 100 g packets of loose leaf tea. The leaves of Vana Tulsi, West Indian lemongrass, East Indian lemongrass and flowers of China rose were collected from the herbal garden of Lovely Professional University, Punjab, India in the month (April, 2017 and May, 2018). These plant species were verified by Dr. Sumeet Gairola, IIIM (Indian Institute of Integrative Medicine) and the voucher specimen were submitted to Janaki Ammal Herbarium of IIIM, Jammu, India. The botanical name, family and accession numbers of the identified herbs are given in the **Table 11**. Herbal teas are also known as tisanes are prepared in the same manner as that of tea. The medicinal plants used as herbal teas investigated in this study are *Ocimum gratissimum* (Vana Tulsi), *Cymbopogon citratus* (West Indian lemongrass), *Cymbopogon flexuosus* (East Indian lemongrass) and *Hibiscus rosa-sinensis* (China rose), investigated to be rich in antioxidants (Naithani et al., 2006; Jadhav et al., 2009).

Table 11: Botanical name, family and accession number of identified herbs used in this study.

| Sample No | Botanical name | Family | Accession number |
|-----------|---|-----------|------------------|
| 1. | <i>Ocimum gratissimum</i> L. | Lamiaceae | RRLH-23391 |
| 2. | <i>Cymbopogon citratus</i> (DC.) Stapf | Poaceae | RRLH-23388 |
| 3. | <i>Cymbopogon flexuosus</i> (Nees ex Steud W. Watson) | Poaceae | RRLH-23389 |
| 4. | <i>Hibiscus rosa-sinensis</i> L. | Malvaceae | RRLH-23393 |

5.2.1 Drying process

The collected leaves and flowers of plants were washed with water and air-dried for three days under shade at room temperature (Liu et al., 2016). The aerial plant parts were cut into minor sections for sample preparation. These plant parts were then stored into the air tight jars for further use.

5.2.2 Sample preparation

5.2.2.1 Preparation of aqueous infusions

The aqueous infusion of green tea (loose leaf), *O. gratissimum* (leaves), *C. citratus* (leaves), *C. flexuosus* (leaves) and *Hibiscus-rosa-sinensis* (flowers) alone or as binary combination with green teas (2% W/V, 1:1) were prepared by soaking them in hot water (95-100 °C) for 5 minutes and then all the samples have been screened using whatman's filter paper (no. 1). Binary combination of green tea and *O. gratissimum* (2% W/V) at different ratios (3:1, 2:1, 1:1, 1:2 and 1:3) were also prepared in the same manner as mentioned above (Perva-Uzunalic et al., 2006).

5.3 Determination of biological properties

The above mentioned infusions were screened for antioxidant potential employing chemical methods (DPPH, ABTS and NO), ex- vivo assays (Lipid peroxidation and haemolysis) and in vivo angiogenic assay (Chick chorioallantoic membrane).

5.3.1 The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity

5.3.1.1 Preparation of standard solution

The required amount of ascorbic acid was dissolved in distilled water obtained different concentrations (2, 4, 6, 8 and 10µg/ml).

5.3.1.2 Preparation of DPPH Solution

The DPPH solution (0.3mM) was dissolved in methanol and was protected from light by wrapping the beaker with aluminium foil.

5.3.1.3 Protocol for assessment of DPPH radical scavenging potential

DPPH radical quenching potential was examined by following the procedure of Mensor et al., 2001 with few modifications. The 0.3mM DPPH solution was formed by dissolving DPPH in methanol. By diluting it with 50 % methanol, the OD (optical density) of the prepared DPPH solution was defined between 0.8-1. The different concentrations of infusions of alone, green tea combinations and ascorbic acid were applied to 2 ml of DPPH solution separately. The purple color changes to the yellow color were thus measured at 520 nm after 30 minutes of the incubation period. The DPPH solution (2ml) taken as control and methanol as blank. The study was conducted in triplicates. The DPPH quenching ability was measured by following relationship:

$$\% \text{ DPPH scavenging potential} = \frac{A_c - A_i}{A_c} \times 100$$

Whereas, A_c = Absorbance of control, A_i = Absorbance of infusion

5.3.2 The [2, 2'- azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)] (ABTS) radical-scavenging activity

5.3.2.1 Preparation of standard solution

The required quantity of standard (Ascorbic acid) was dissolved in water to give the concentrations of 2, 4, 6, 8 and 10µg/ml.

5.3.2.2 Preparation of ABTS Solution

The ABTS solution (7mM) dissolved in 10ml distilled water and sodium persulphate solution (2.4 mM) in 10 ml distilled water, both were mixed in 1: 1 ratio.

5.3.2.3 Protocol for estimation of ABTS scavenging capacity

This assay relies on the capacity of various substances to scavenge radical ABTS. The radical was prepared by combining ABTS stock solution (7 mM) with a 1:1 ratio of potassium persulfate (2.4 mM). The mixture was kept in the dark at room temperature for 16 h until the completion of the reaction. The ABTS solution's optical density (OD) was set between 0.8-1 by diluting it with 50 % methanol. Different concentrations of all infusions alone, green tea combinations and ascorbic acid were added separately to 2ml of ABTS solution (Re et al., 1999). After half an hour of incubation, the absorbance was taken at 745nm of all respective samples. The ABTS scavenging activity of test samples were determined as follow:

$$\% \text{ABTS scavenging potential} = \frac{A_c - A_i}{A_c} \times 100$$

Whereas, A_c = Absorbance of control, A_i = Absorbance of infusion

5.3.3 Nitric oxide scavenging assay (NO)

5.3.3.1 Preparation of standard solution

In distilled water, the required amount of Ascorbic acid was dissolved to give the concentrations of 2, 4, 6, 8 and 10 μ g/ml.

5.3.3.2 Preparation of phosphate buffer saline (PBS)

Phosphate buffer was made by dissolving 0.8g of sodium chloride (NaCl), 0.02g of potassium chloride (KCl), 0.144g of disodium phosphate (Na_2HPO_4) and 0.024g of potassium dihydrogen phosphate (KH_2PO_4) in 80 ml of distilled water. Then adjusted the pH 7.4 by pH meter and make total volume 100ml by adding distilled water.

5.3.3.3 Preparation of sodium nitroprusside solution

The sodium nitroprusside (10mM) was prepared by dissolving 0.131g of sodium nitroprusside in phosphate buffer.

5.3.3.4 Preparation of Griess reagent

Griess reagent was prepared by dissolving 1g of sulphanilamide, 0.1g of naphthyl ethylenediamine dihydrochloride (NEDA) and 2ml of O- phosphoric acid in 100ml of distilled water.

5.3.3.5 Protocol for estimation of NO radical scavenging capacity

Following the method defined by Shirwaikar et al., 2006, the NO scavenging ability was studied. Different concentrations of both infusions alone, green tea combinations and ascorbic acid is calculated to have been dissolved in a pH 7.4 phosphate buffer and incubated with sodium nitroprusside (10 mM) in a regular 37 °C phosphate buffer for 30 minutes, respectively. Following incubation, 0.5 ml of Griess reagent (1% sulphanilamide, 2% O-phosphoric acid and 0.1% naphthyl ethylenediamine dihydrochloride) was applied to the test tubes. At 546 nm, the absorbance was assessed. Nitric oxide scavenging potential of test samples was determined by the formula given below.

$$\% \text{ NO scavenging activity} = \frac{A_c - A_i}{A_c} \times 100$$

Whereas, A_c = Absorbance of control, A_i = Absorbance of infusion

5.3.4 Anti-Lipid peroxidation assay

5.3.4.1 Preparation of standard solution

The required quantity of butylated hydroxytoluene was dissolved in methanol to give the concentrations of 10, 20, 30, 40 and 50µg/ml.

5.3.4.2 Preparation of FeSO₄

FeSO₄ (15 mM) was prepared by dissolving 0.082g FeSO₄ in 20ml of distilled water.

5.3.4.3 Preparation of TBA:TCA:HCl mixture

TBA:TCA:HCl mixture was made by dissolving 0.5g of TBA, 20 ml of TCA and 0.5ml of HCl in distilled water and make the total volume 100ml.

5.3.4.4 Protocol for estimation of anti- lipid peroxidation activity

From the slaughterhouse, fresh chicken liver was collected, preserved in cold or chilled PBS and held at 4°C until use. The liver homogenate (10%) was made by using homogenizer and centrifuged at 2000 rpm. For lipid peroxidation estimation, the supernatant was taken. The mixture of liver homogenate, different concentrations of various infusions alone, green tea combinations and BHT, were added separately to 15mM of FeSO₄ at 37°C for half an hour. A 2ml of TBA, TCA and HCl (1:1:1) mixture was added after incubation and held in a water bath for 20 minutes at 100°C. Then it was cooled, centrifuged and supernatant absorption was

estimated at 532nm (Ohkawa et al., 1979). The inhibition percentage was determined by comparing the test outcome with the controls as shown below:

$$\% \text{ Scavenging activity} = \frac{Ac - Ai}{Ac} \times 100$$

Whereas, Ac = Absorbance of control, Ai = Absorbance of infusion

5.3.5 Anti- haemolytic assay

5.3.5.1 Preparation of standard solution

In distilled water, the required amount of ascorbic acid was dissolved to give the concentrations of 2, 4, 6, 8 and 10µg/ml.

5.3.5.2 Protocol for inhibition of RBC haemolysis

Blood samples were obtained in a heparin-coated tube from adult males and females aged 23-26 years. A written agreement has been received from the blood donors. At 1500 rpm, the collected blood was centrifuged for 10 min. Erythrocytes were washed with cold saline phosphate (pH 7.4) three times and centrifuged for 10 min at 1500 rpm. A portion of erythrocytes was delivered into a tube and various infusions alone were applied to different tubes, binary combinations and ascorbic acid, accompanied by 100 mM hydrogen peroxide to cause haemolysis, and incubated at 37 °C for 3 hours. A 2 ml of phosphate buffer saline was inserted into each tube after incubation, and these tubes were centrifuged for 10 minutes at 5000 rpm. (Okoko and Ere, 2012). At 540 nm, the absorbance was measured. More haemolysis is demonstrated by the rise in absorbance.

$$\% \text{ Inhibition activity} = \frac{Apc - Ai}{Apc} \times 100$$

Whereas, Apc = Absorbance of positive control and Ai = Absorbance of infusion

5.3.6 Estimation of total phenolic content (TPC)

5.3.6.1 Preparation of standard solution

The required amount of gallic acid was dissolved in water to give the concentrations of 10, 20, 30, 40, 50 and 60µg/ml.

5.3.6.2 Preparation of Folin-Ciocalteu reagent

Folin-Ciocalteu reagent (0.2N) was dissolved in distilled water.

5.3.6.3 Preparation of 20% sodium carbonate (Na₂CO₃)

The sodium carbonate (20%) solution was prepared by dissolving in distilled water.

5.3.6.4 Protocol for TPC

Using Folin-Ciocalteu reagent, TPC was measured in various infusions (Singleton et al., 1999). About 1ml of distilled water and 75 µl of Folin–Ciocalteu reagent (0.2 N) were added following by addition of 25 µl of all infusions alone and combinations, and mixed. After five minutes, 375 µl of 20% sodium carbonate (Na₂CO₃) added and well blended. These samples were incubated at room temperature for 120 minutes in the dark. The optical density (OD) was estimated at 760 nm. The findings were expressed as mg gallic acid equivalents (GAE) per gram.

5.3.7 Estimation of total flavonoid content (TFC)

5.3.7.1 Preparation of standard solution

The required amount of quercetin was dissolved in water to give the concentrations of 10, 20, 30, 40 and 50µg/ml.

5.3.7.2 Preparation of 10% Aluminum chloride solution

Aluminum chloride (10%) was prepared by dissolving aluminum chloride in distilled water.

5.3.7.3 Preparation of 30% ethanolic solution

30ml of ethanol was added to 70ml of distilled water

5.3.7.4 Preparation of 5% sodium nitrite solution

The sodium nitrite (5%) solution was prepared by dissolving sodium nitrite in water

5.3.7.5 Protocol for TFC

Total flavonoids content was calculated by using an Aluminum chloride colorimetry test (Zhishen et al., 1999). A 250 µl infusions alone and in combination blended with 1.5 ml of ethanol and 120µl of sodium nitrite solution. A 120µl of aluminium chloride solution (10 %) was applied to the above solution after five minutes and kept as such for five minutes. After that 0.5ml of 1M NaOH solution was also added. The solution prepared above was incubated

at 37 °C for 30 min and absorbance was measured at 415 nm. The TFC was expressed as mg quercetin equivalents (QE) per g dry weight.

5.4 Methods to determine the interaction types

The interaction (synergistic, antagonistic or additive) between green tea and other plants (Vana Tulsi, West Indian lemongrass, East Indian lemongrass and China rose) was determined using EC₅₀ (Effective concentration causing 50% scavenging activity), combination index (CI), isobolograms and polygonograms (Chou, 2006), applying CmpuSyn software.

5.4.1 Isobolographic, Combination index (CI), and Polygonogram analysis

5.4.1.1 Isobologram: The dose effect of two phytochemicals or mixtures is illustrated by the Isobologram (Efferth and Koch, 2011). Isobologram a graphical representation composed of individual drug doses such as dose A represents the EC₅₀ of one drug and dose B stands for EC₅₀ of another drug. A line shown between X and Y axes joining points of their respective EC₅₀ values (the line of additivity). If the EC₅₀ of the combination lies below the line of additivity it indicates synergistic interaction, above the line antagonistic interaction and on this line, is the additive effect (Tallarida, 2001).

5.4.1.2 Combination index (CI): CI value tells about the type of interaction in a binary mixture. If CI value is less than 1 (CI < 1) it is synergistic interaction and if greater than 1 (CI > 1) it is antagonistic interaction and if equal to 1 (CI = 1) demonstrated additive interaction. The range of CI value and the type of interaction was followed as given: < 0.1 (very strong synergism), 0.1-0.3 (strong synergism), 0.3-0.7 (synergism), 0.7-0.85 (moderate synergism), 0.85- 0.90 (slight synergism), 0.90-1.10 (nearly additive), 1.10-1.20 (slight antagonism), 1.20-1.45 (moderate antagonism), 1.45-3.3 (antagonism), 3.3-10 (strong antagonism) and > 10 (very strong antagonism) (Chou, 2006).

5.4.1.3 Polygonogram: It is a pictorial representation illustrating antagonism, additive and synergism for comparing two drug combinations (Chen et al., 2019). All of the above methods rely on each drug's dose effect curve, where the EC₅₀ value is determined to define the form of interaction.

5.4.2 The ATR-FTIR (Attenuated total reflectance-fourier transforms infrared spectroscopy)

FTIR spectroscopy was used to detect the existing functional groups and the intermolecular interactions between green tea and other herbs. FTIR spectra was obtained using mid infrared region (MIR) corresponding to 600-4000cm⁻¹ with 15 scans for each spectral collection using

Shimadzu spectrometer 8400S (Mandal and Bhattacharya, 2015). FTIR of all individual samples, combinations and different ratios were carried out.

5.4.3 Ultraviolet–visible (UV-VIS) analysis

The ultraviolet–visible spectra of all samples were analyzed between the ranges of 200-600nm employing UV- visible spectrophotometer to further determine the concentration of the phytochemicals in these infusions.

5.5 *In- vivo* modulation of angiogenesis employing chick chorioallantoic membrane (CAM) assay

5.5.1 Preparation of Nacl

Nacl (0.9%) were prepared by dissolving in distilled water.

5.5.2 Chick chorioallantoic membrane (CAM) assay

The anti-angiogenic effect of different extracts such as GT, OG, CC, CF, HR, GT+OG, GT+CC, GT+CF and GT+HR were examined by using chick chorioallantoic membrane (CAM) assay as described previously with some modifications (Fett et al., 1985). The fertilized eggs were obtained from the local egg poultry farm from Jalandhar. The eggs were cleaned with 70% ethanol and placed in an incubator at 37⁰C with approximately 60-65% humidity. On the second day of incubation, 2-2.5ml of albumin was taken out from the eggs so that the developed embryo will not attach to the egg shell. On the eighth day of incubation, the egg shells were cut open from the wide end. There were investigated twenty experimental groups with three eggs per group.

- (i) 0.9% Nacl control (w/v)
- (ii) 200ng VEGF/embryo
- (iii) 5µg/ml GT/embryo
- (iv) 200ng VEGF/embryo plus 5µg/ml GT
- (v) 5µg/ml OG/embryo
- (vi) 200ng VEGF/embryo plus 5µg/ml OG
- (vii) 5µg/ml CC/embryo
- (viii) 200ng VEGF/embryo plus 5µg/ml CC
- (ix) 5µg/ml CF /embryo
- (x) 200ng VEGF/embryo plus 5µg/ml CF
- (xi) 5µg/ml HR /embryo
- (xii) 200ng VEGF/embryo plus 5µg/ml HR
- (xiii) 5µg/ml GT+OG /embryo

- (xiv) 200ng VEGF/embryo plus 5µg/ml GT+OG
- (xv) 5µg/ml GT+CC /embryo
- (xvi) 200ng VEGF/embryo plus 5µg/ml GT+CC
- (xvii) 5µg/ml GT+CF /embryo
- (xviii) 200ng VEGF/embryo plus 5µg/ml GT+CF
- (xix) 5µg/ml GT+HR/embryo
- (xx) 200ng VEGF/embryo plus 5µg/ml GT+HR

The NaCl, VEGF (vascular endothelial growth factor) and various extracts (5µg/ml) were impregnated on circular filter paper discs on the CAM between the two mature blood vessels of eight day old embryos. Now the opened window on the egg shell was sealed with parafilm or tape. The whole experiment was carried out under sterile conditions. After 24 hours of incubation the tape was removed and CAM was exposed and photographed. The average vessel length was counted by using AngioTool software.

5.6 The cell Culture

A medium RPMI 1640 containing 10 % fetal bovine serum and L-glutamine (2 mM) was developed for the lung cancer cell line (A549). In 96, well microtiter plates at 100 µl plating densities cells were inoculated for cytotoxicity screening of present experiment. During inoculation of cells, incubate the plates for 24h before the implementation of experimental drugs at 95 % air, 5 % CO₂, 37° C and 100 % relative humidity.

5.6.1 Cytotoxicity assay by Sulforhodamine-B (SRB)

The cytotoxic effect of different samples was determined by using SRB (Sulforhodamine-B) assay (Skehan et al., 1990). In the beginning of the experiment the drugs were solubilized in 100mg/ml of DMSO (dimethyl sulfoxide) and diluted with water to 1mg/ml and kept frozen until further procedure. A frozen concentrate aliquote (1mg/ml) was defrosted at the time of drug addition that was diluted to 100, 200, 400 and 800µg/ml with a full medium containing test item. The volume (10µl) of various sample dilutions have already been applied to respective microtiter wells containing 90 µl of medium, thus resulting in the final concentration of the product (10, 20, 40 and 80 µg/ml).

Incubate the plates after the addition of compounds for 48 hours under standard conditions and the test was finished by adding cold TCA. By mixing 50 µl of 30 % cold (w/v) TCA (final concentration, 10 % TCA), the cells were fixed *in situ* and incubated for an hour at

4°C. Drain the supernatant and wash the plates with tap water for 5 minutes then air dried. The 50 µl of SRB solution at 0.4 % (w/v) in 1 % acetic acid added in wells and incubated for 20 minutes at room temperature. The unbound dye was recovered after staining and the residual dye was removed by washing the plates 5 times with 1 % acetic acid, then air dried. The unavoidable stain was then detached with a base of 10 mM trizma base and the absorbance was read at 540 nm and 690 nm with reference wavelength.

The average growth for test wells was measured on a plate-by-plate basis compared with control wells. The percent growth was measured by the following formula:

$$\text{Percent growth} = \text{Absorbance of sample} / \text{Absorbance of control} \times 100$$

The percentage increase was evaluated at each concentration of the drug using six measurements for absorbance [time zero (Tz), control growth (C), and test growth in the presence of product at 4 concentration levels (Ti)]. The inhibition of percentage growth was measured as:

$$\text{Ti/C} \times 100 \%$$

The images were taken for culture at highest concentration i-e 80µg/ml of all samples.

5.7 Assessment of cancer chemopreventive potential of green tea, *O. gratissimum* and their combination against B[a]P mediated lung carcinogenesis in mice model

5.7.1 Preparation of infusions

The aqueous infusions of green tea and *O. gratissimum* (1%) alone and their binary combination (1:1) were steeped at 95-100 °C, for 5 minutes (Farooq and Sehgal, 2019). Using Whatman's filter paper no.1, these infusions were filtered separately. These infusions were prepared daily till the end of the experiment.

5.7.2 Preparation of benzo(a)pyrene

B(a)P was dissolved in corn oil (72 mg/ml).

5.7.3 Animals

Male Swiss albino mice (*Mus musculus*) of 16-18 weeks were obtained from NIPER (National Institute of Pharmaceutical Education & Research), Mohali, Punjab. After arrival of animals in our animal facility, they were kept in cages bedded with rice husk. Prior to the beginning of the experiment, the animals were allowed to acclimatize for 15 days. The mice were kept in an air-conditioned room with humidity 50-60% in a regular period of light and darkness alternating for 12 hours. All mice were fed with a standard pellet diet (Ashirwad Industries, Ropar, Punjab, India) and water *ad libitum*. This research was carried out in compliance with ethical principles accepted by the institutional animal ethics committee (IAEC) (Registration No.: 954/PO/Re/S/06/CPCSEA). The water, food and infusion consumption were measured daily and the body weight was monitored after every week during the whole experimental period.

5.7.4 Experimental setup

A total of 30 mice were distributed with 6 mice per group, in five groups. The Group I animals as vehicle control (intraperitoneal injection of corn oil), Group II positive control (i.p, 100 mg/Kg, BW of B(a)P in corn oil), Group III (oral dose of GT infusion), Group IV (oral dose of OG infusion) and Group V (oral dose of GT and OG combination). The animals in Group I and Group II were fed with water and in Gp. II to Gp.V were administered a single i.p dose of B(a)P in 0.2ml of corn oil. In Gp.III, Gp. IV and Gp.V, the animals were administered oral infusion of 1% green tea (GT), 1% *Ocimum gratissimum* (OG) or 2% their combination (GT+OG) as the primary source drinking water for 15 days before that of B[a]P dose and continued up to termination as depicted in **Fig. 16** (Katiyar et al., 1993; Yan et al., 2005; Kamaraj et al., 2009; Ravichandran et al., 2011). Five groups of animals were maintained for whole experiment as below:

Group I: Vehicle control administered with corn oil.

Group II: Benzo(a)pyrene administered group.

Group III: Green tea and benzo(a)pyrene

Group IV: *Ocimum gratissimum* and benzo(a)pyrene

Group V: (Green tea and *O. gratissimum* combination) and benzo(a)pyrene

The experimental animals fasted overnight and then killed using mild anesthesia after 22th week of B(a)P dose. The lungs of all animals were extracted from all groups.

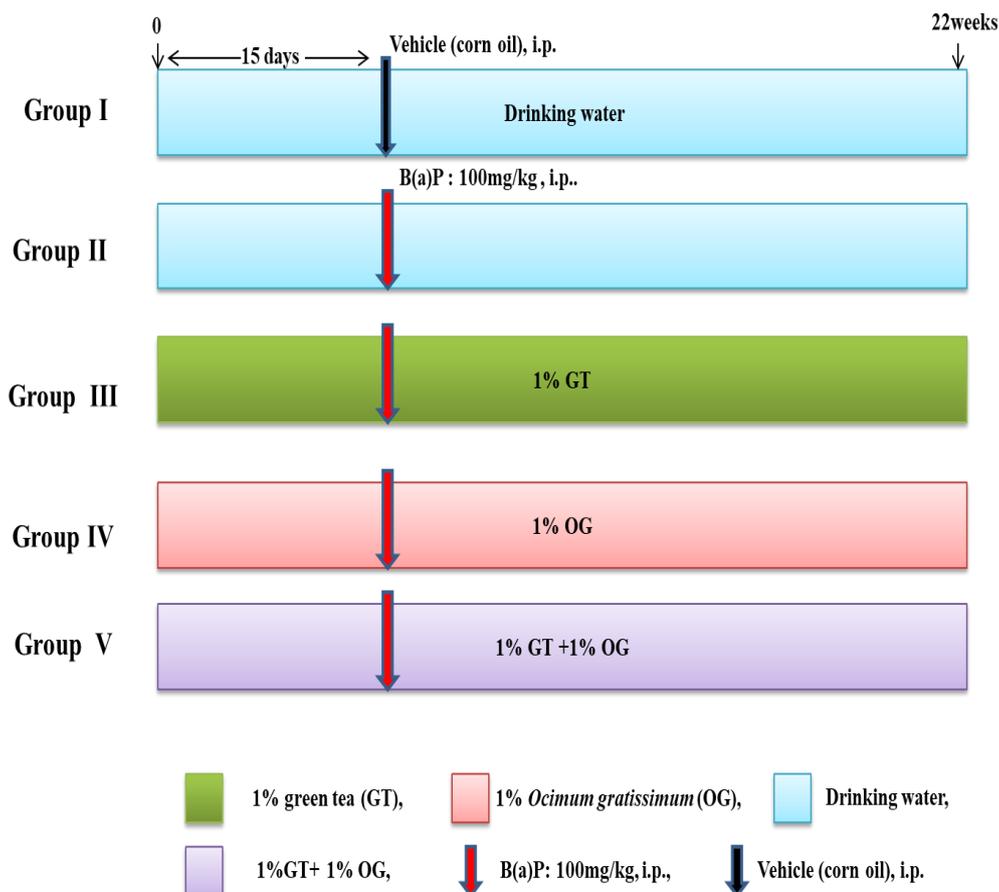


Fig. 16: Schedule of animal care to study the effect of aqueous infusion of GT, OG and GT+OG against B(a)P induced lung lesions.

5.7.5 Biochemical analysis

The blood samples were obtained by direct heart puncture of anaesthetized mice into heparin-containing eppendorf tubes. By using centrifugation at 2000 rpm for 10 minutes, the plasma was obtained. The plasma taken was then stored at -20°C for DPPH and ABTS assays.

5.7.5.1 DPPH radical scavenging potential

According to Mansouri et al., 2005, the ability of mice plasma to scavenge the free radical DPPH was assessed. The DPPH (0.3mM) was prepared by dissolving DPPH in methanol. The DPPH solution's OD (optical density) was set within the 0.8-1 range by diluting it with 50 % methanol. A volume of 20 μl of plasma from all groups of animals was added separately to 2ml of DPPH solution. The absorbance of this solution was detected at 517nm, after incubation of 30 minutes at room temperature. DPPH solutions without plasma were used as the control.

5.7.5.2 ABTS radical scavenging activity

Mice plasma ability to scavenge the free radical ABTS was measured. By mixing ABTS (7mM) stock solution with (2.4 mM) potassium persulfate in a 1:1 ratio, the ABTS radical cation was prepared. At room temperature, this mixture was preserved in dark for 16 h till the reaction is completed. The OD (optical density) of ABTS solution was set between the range of 0.8-1 by diluting it with 50% methanol. A volume of 20 μ l of plasma from all groups of animals was added to 2ml of ABTS solution separately (Re et al., 1999). After incubation of 30 minutes, the absorbance of respective samples was measured at 745nm.

5.7.6 Histopathological evaluation

Lung tissues were extracted and weighed at the end of the experiment (22nd week) and put in phosphate buffer saline (pH 7.4) for 24hours, and then fixed in 10 % formaldehyde. At increasing ethanol concentrations, the lung tissues were dehydrated, cleaned in xylene, and fixed in paraffin to create the block. The serial sections (5 μ m) of left lung were cut and stained with hematoxylin and eosin for microscopical observation and photographs were captured. Two slides of each mouse from all groups were analyzed under microscope.

5.8 Statistical analysis

All experiments were performed in triplicates and reported as mean \pm standard deviation (SD) for each outcome and for six animals in each group. The one way analysis of variance (ANOVA) study was used to conduct the statistical analysis, followed by Tukey's honestly significant difference test using SPSS software (version 18). The findings were considered statistically important if the p-values were 0.05 or less.

CHAPTER 6

RESULTS

RESULTS

6.1 Antioxidant activity of green tea or herbal teas and their combinations

The antioxidant potential is an important tool in evaluating the ability of plant extracts or phytochemicals to neutralize the reactive species implicated with many diseases. The aqueous infusions of green tea (GT), *O. gratissimum* (OG), *C. citratus* (CC), *C. flexuosus* (CF) and *H. rosa-sinensis* (HR) alone, and the binary combination of green tea with these plants at a constant ratio (1:1) was estimated for antioxidant capacity employing chemical assays like DPPH, ABTS and NO and *ex-vivo* tests such as chick lipid peroxidation and erythrocyte haemolysis. In all the performed antioxidant assays, EC₅₀ value of each aqueous infusion was calculated. The lower the EC₅₀ value the higher is the antioxidant activity of the infusion. The total phenolic and flavonoid content of different aqueous infusions were also evaluated using gallic acid and quercetin as standards.

6.1.1 DPPH assay

DPPH is a stable free radical that is commonly used as a model system to examine the antioxidant capacity of different extracts. The EC₅₀ value of different aqueous infusions is based upon the scavenging activity. The infusions such as GT, GT+OG, GT+CC, GT+CF and GT+HR demonstrated similar ($p \geq 0.05$) scavenging ability which is much higher ($p \leq 0.05$) in comparison to HR, OG, CF and CC respectively, as depicted in **Fig. 17** and **Table 12**. Ascorbic acid (Vitamin C) is used as a standard showed higher DPPH radical quenching potential and lower EC₅₀ value as compared to all other samples.

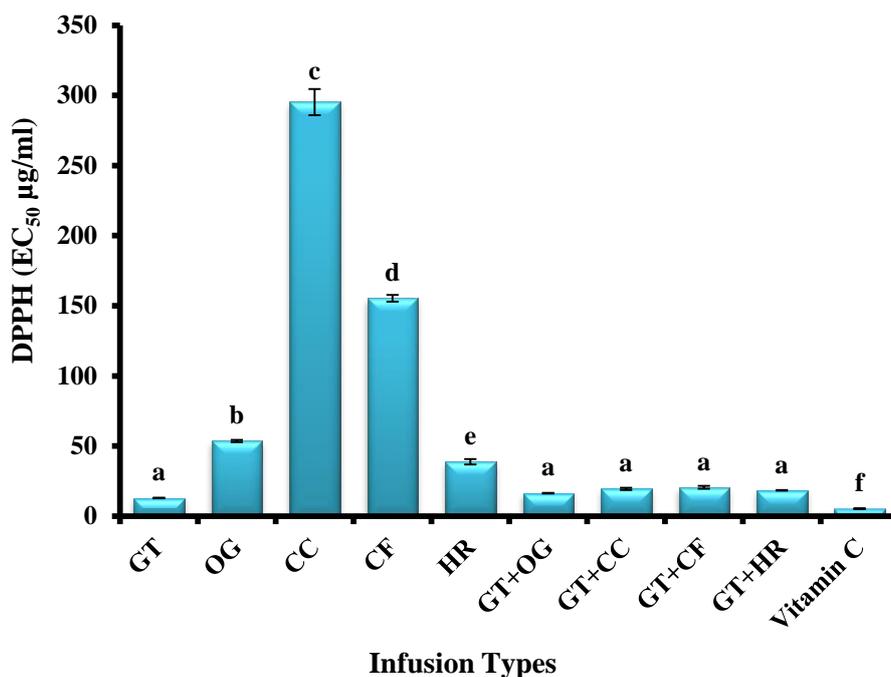


Fig. 17: DPPH scavenging activity of Green tea (GT), *O. gratissimum* (OG), *C. citratus* (CC), *C. flexuosus* (CF), *H. rosa-sinensis* (HR), Vitamin C and green tea combinations. The data are shown as MEAN \pm S.D of 3 independent tests (each with triplicates for each test point). EC₅₀(Effective concentration causing 50% scavenging activity). The dissimilar alphabets illustrated significant difference ($p \leq 0.05$) among the EC₅₀ of different infusion types.

6.1.2 ABTS assay

ABTS is an unstable colored free radical and can be dissolved both in aqueous and organic phases. The ABTS radical scavenging activity was found in the following descending order: GT+OG, GT+CC, GT, GT+HR, GT+CF, HR, OG, CC and CF, respectively as given in **Fig. 18**. The ABTS radical scavenging efficacy of ascorbic acid (vitamin C) was observed to be comparable ($p \geq 0.05$) to GT, HR, GT+CC, GT+CF, GT+HT infusions, and the least activity was of CF ($p \leq 0.05$) infusion as given in **Table 12**.

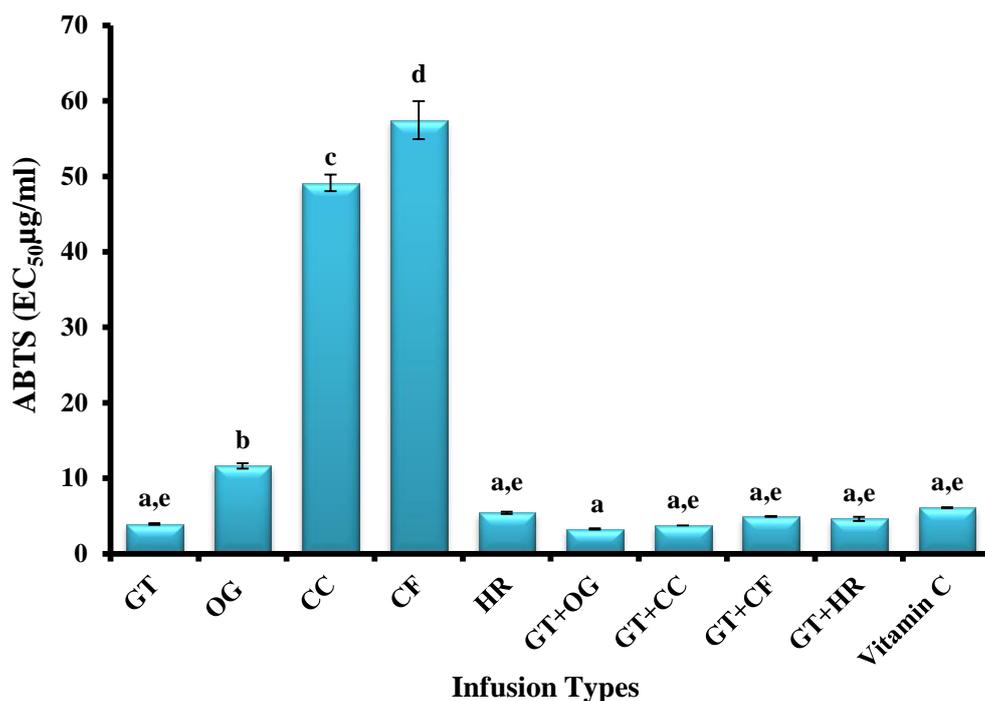


Fig. 18: ABTS scavenging activity of Green tea (GT), *O. gratissimum* (OG), *C. citratus* (CC), *C. flexuosus* (CF), *H. rosa-sinensis* (HR), Vitamin C and green tea combinations. The data are shown as MEAN \pm S.D of 3 independent tests (each with triplicates for each test point). EC₅₀(Effective concentration causing 50% scavenging activity). The dissimilar alphabets illustrated significant difference ($p \leq 0.05$) among the EC₅₀ of different infusion types.

6.1.3 NO assay

Nitrite quenching ability of different infusions was tested employing NO assay and the results are depicted in the **Fig. 19**. GT+OG, GT+CC, GT+HR, GT+CF and GT illustrated similar activity ($p \geq 0.05$) that is higher ($p \leq 0.05$) than OG, CF, HR and CC, respectively. The standard (Vitamin C) revealed similar nitric oxide radical scavenging activity as that of GT+OG ($p \geq 0.05$) **Table 12**.

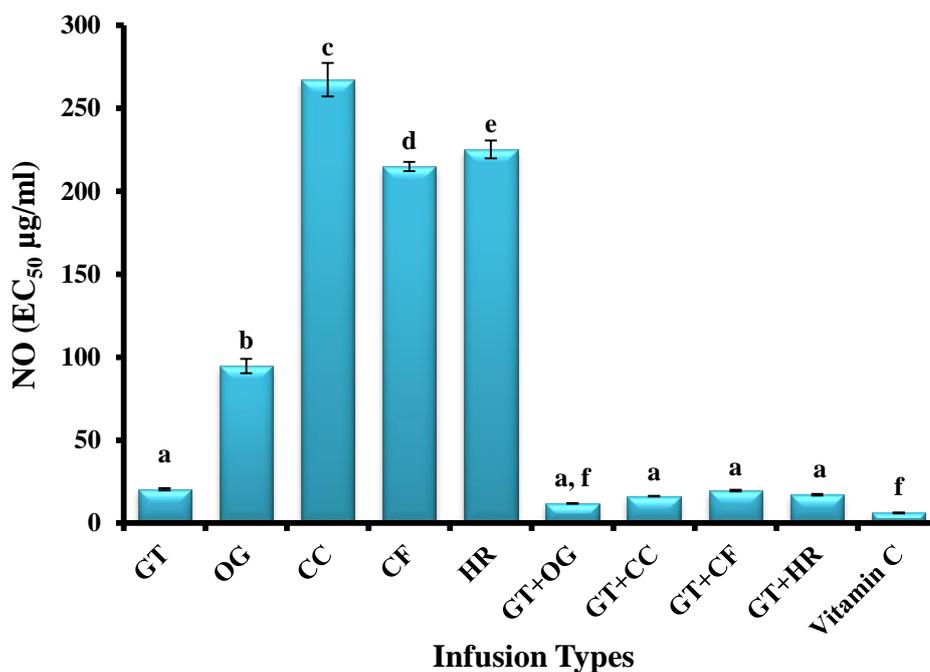


Fig. 19: Nitric oxide scavenging activity of Green tea (GT), *O. gratissimum* (OG), *C. citratus* (CC), *C. flexuosus* (CF), *H. rosa-sinensis* (HR), Vitamin C and green tea. The data are shown as MEAN \pm S.D of 3 independent tests (each with triplicates for each test point). EC₅₀(Effective concentration causing 50% scavenging activity). The dissimilar alphabets illustrated significant difference ($p \leq 0.05$) among the EC₅₀ of different infusion types.

6.1.4 Anti-lipid peroxidation assay

The inhibitory action of different aqueous infusions was determined against FeSO₄-induced lipid peroxidation in chick liver. The butylated hydroxytoluene (BHT) used as standard demonstrated highest ($p \leq 0.05$) decline in lipid peroxidation. GT+OG combination revealed maximum ($p \leq 0.05$) anti-lipidperoxidation activity among all the infusion (GT+CC, GT+HR, GT+CF, GT, CF, CC, HR and OG) examined, while OG demonstrated the lowest activity as given in **Fig. 20** and **Table 12**.

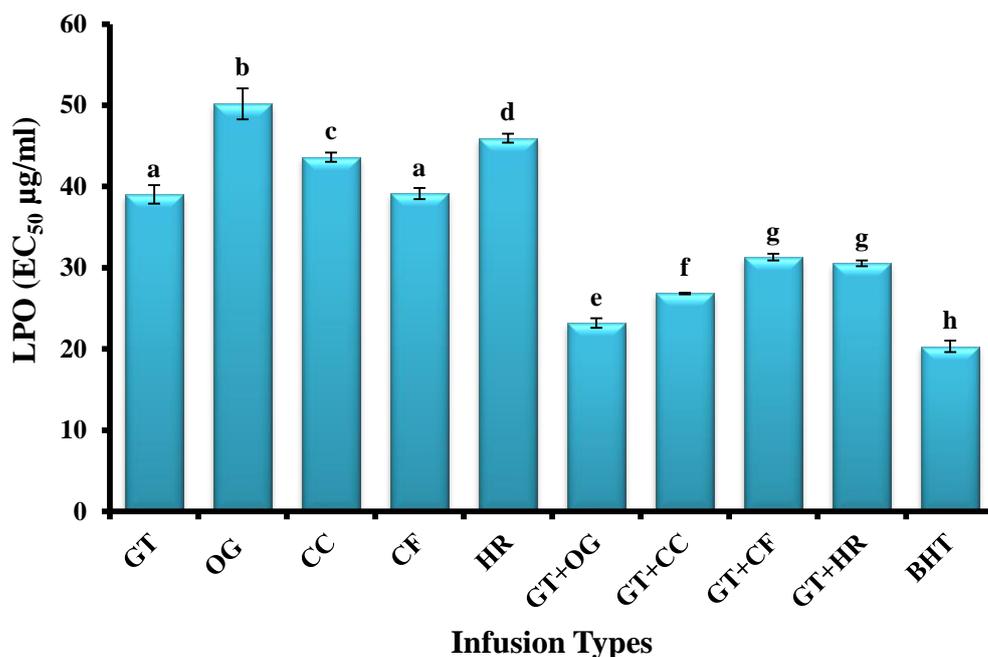


Fig. 20: Anti-lipidperoxidation activity of Green tea (GT), *O. gratissimum* (OG), *C. citratus* (CC), *C. flexuosus* (CF), *H. rosa-sinensis* (HR), Butylated hydroxytoluene (BHT) and green tea combinations. The data are shown as MEAN \pm S.D of 3 independent tests (each with triplicates for each test point). EC₅₀(Effective concentration causing 50% scavenging activity). The dissimilar alphabets illustrated significant difference ($p \leq 0.05$) among the EC₅₀ of different infusion types.

6.1.5 Anti-haemolytic assay

Human erythrocytes are used a model system to evaluate the effect of plant extracts against the H₂O₂ - induced haemolysis. GT and GT+OG displayed similar ($p \geq 0.05$) but highest anti-haemolytic activity as compared to other infusions (GT+HR, GT+CF, GT+CC, HR, OG, CF and CC) and standard (Vitamin C) ($p \leq 0.05$) as displayed in **Fig. 21** and **Table 12**.

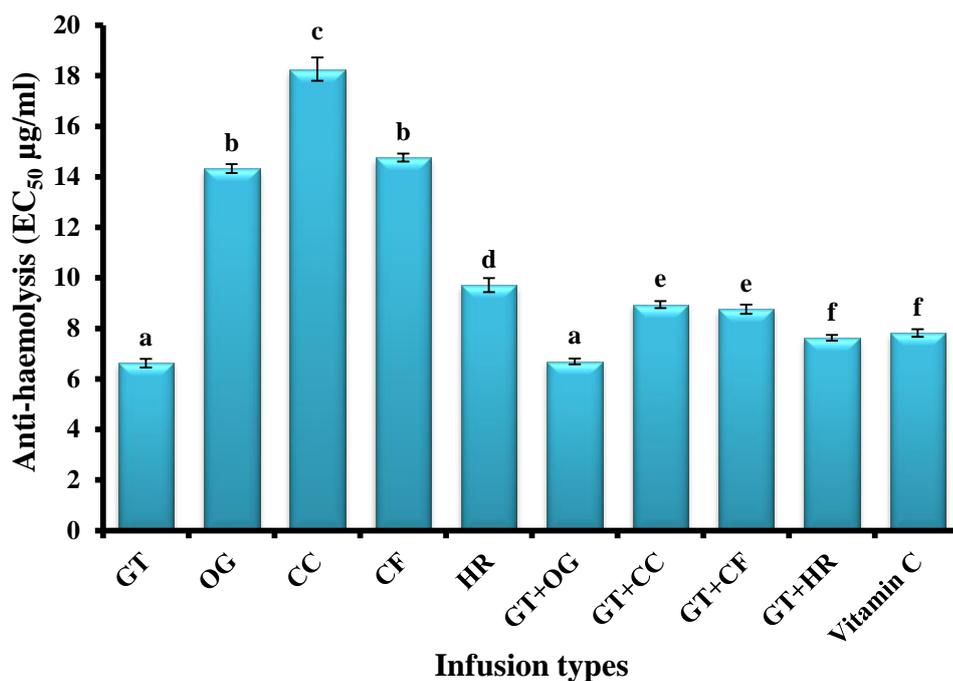


Fig. 21: Anti-haemolytic activity of Green tea (GT), *O. gratissimum* (OG), *C. citratus* (CC), *C. flexuosus* (CF), *H. rosa-sinensis* (HR), Vitamin C and green tea combinations. The data are shown as MEAN \pm S.D of 3 independent tests (each with triplicates for each test point). EC₅₀(Effective concentration causing 50% scavenging activity). The dissimilar alphabets illustrated significant difference ($p \leq 0.05$) among the EC₅₀ of different infusion types.

6.2 The antioxidant interactions between green tea and some medicinal plants

The combination index (CI) value tells about the type of interaction in a binary mixture. The antioxidant interactions of GT and medicinal plants (1:1) were examined as described by Chou (2006), in order to analyze their potential additive, synergistic and antagonistic effects. The range of CI value and the type of interaction followed are given as: < 0.1 (very strong synergism), 0.1-0.3 (strong synergism), 0.3-0.7 (synergism), 0.7-0.85 (moderate synergism), 0.85- 0.90 (slight synergism), 0.90-1.10 (nearly additive), 1.10-1.20 (slightly antagonism), 1.20-1.45 (moderate antagonism), 1.45-3.3 (antagonism), 3.3-10 (strong antagonism) and > 10 (very strong antagonism). In binary combinations of green tea with other plants, synergistic to additive interactions were observed at EC₅₀ in various antioxidant assays. The CI values of all the samples in the performed assays ranged from (0.35-1.00) are given in **Table 12**. The isobolographic analysis for various GT combinations was also carried out. Isobologram is a graphical representation composed of individual drug doses and commonly a straight line of additivity is employed to determine the drug interactions, if the EC₅₀ value is

below this line indicates synergism, above it shows antagonistic interaction, and on or near the line of additivity demonstrate additive effect (Ooko et al., 2017).

6.2.1 Interactions by combination index and isobolographic method

In DPPH test, the CI value of various GT combinations at EC_{50} is displayed in **Table 12**. The binary combinations, GT+OG, GT+CC and GT+CF revealed synergistic interaction whereas GT+HR combination illustrated the additive interaction. The isobolograms demonstrated that for GT+CC, GT+OG, GT+CF combinations, the EC_{50} value lies below the line of additivity indicates synergism (**Fig. 22a, b and c**), and this value touches the line of additivity that shows the additive effect for GT+HR combination (**Fig. 22d**).

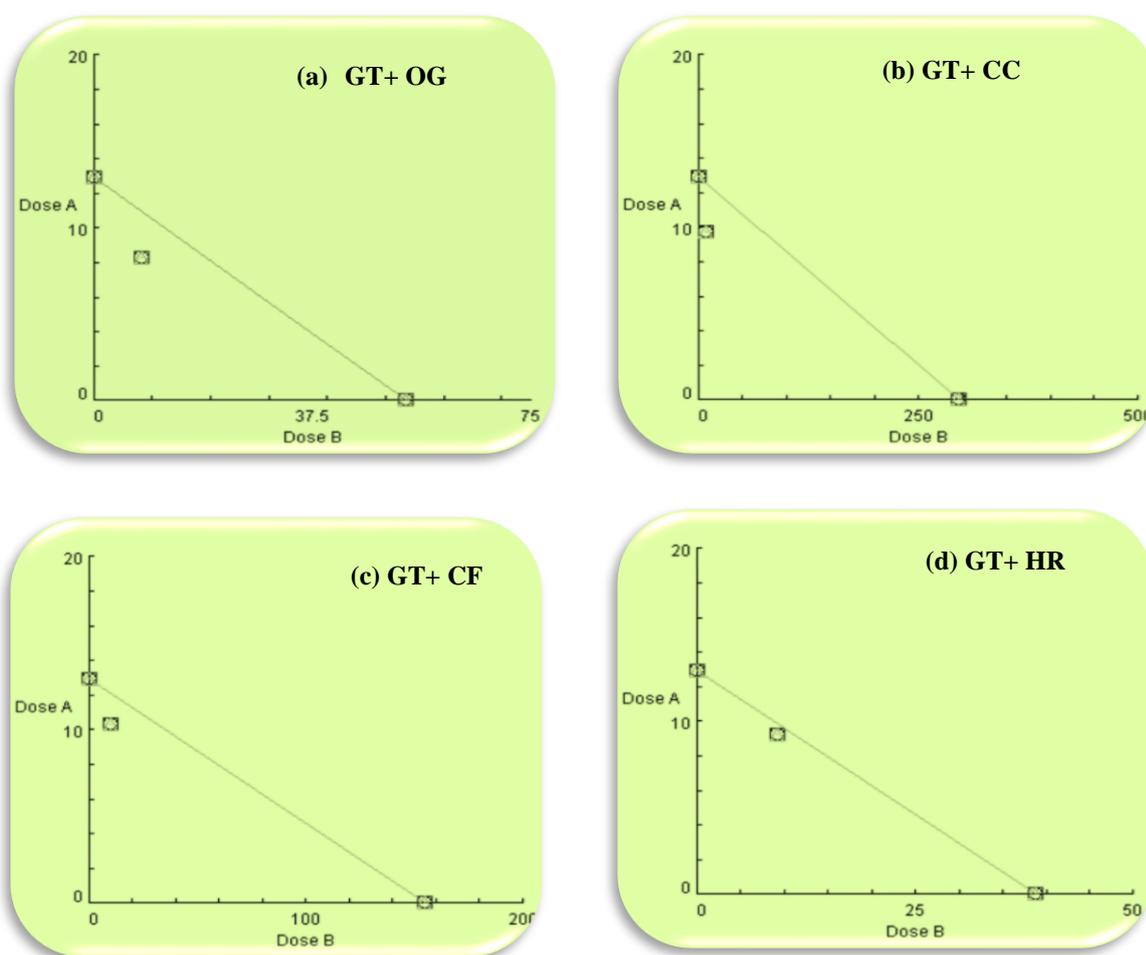


Fig. 22: Isobolograms showing interaction between (a) Green tea and *O. gratissimum* (GT+OG), (b) Green tea and *C. citratus* (GT+CC), (c) Green tea and *C. flexuosus* (GT+CF) and (d) Green tea and *H. rosa-sinensis* (GT+HR) at 1:1 ratio in DPPH assay. Dose A represents the EC_{50} of GT in each graph and Dose B stands for EC_{50} of OG or CC or CF or HR. The straight line (additive line) is made by dose A and B (EC_{50} value). If the square symbol lies below the line of additivity it indicates synergistic interaction, above the line antagonistic and on this line, is the additive effect. The data are shown as

MEAN \pm S.D for 3 independent experiments (each with three replicates of each test point).

In ABTS assay green tea combinations, GT+OG, GT+CC and GT+CF exhibited synergistic interactions whereas GT+HR manifested additive interaction at EC₅₀ as given in **Table 12**. The GT+OG and GT+CC demonstrated similar ($p \geq 0.05$) CI value and maximum synergism as compared to GT+CF. In GT+OG, GT+CC and GT+CF, the EC₅₀ value lies below the line of additivity showed synergism but in case of GT+HR it lies on the line of additivity displaying the additive interaction (**Fig. 23a-d**).

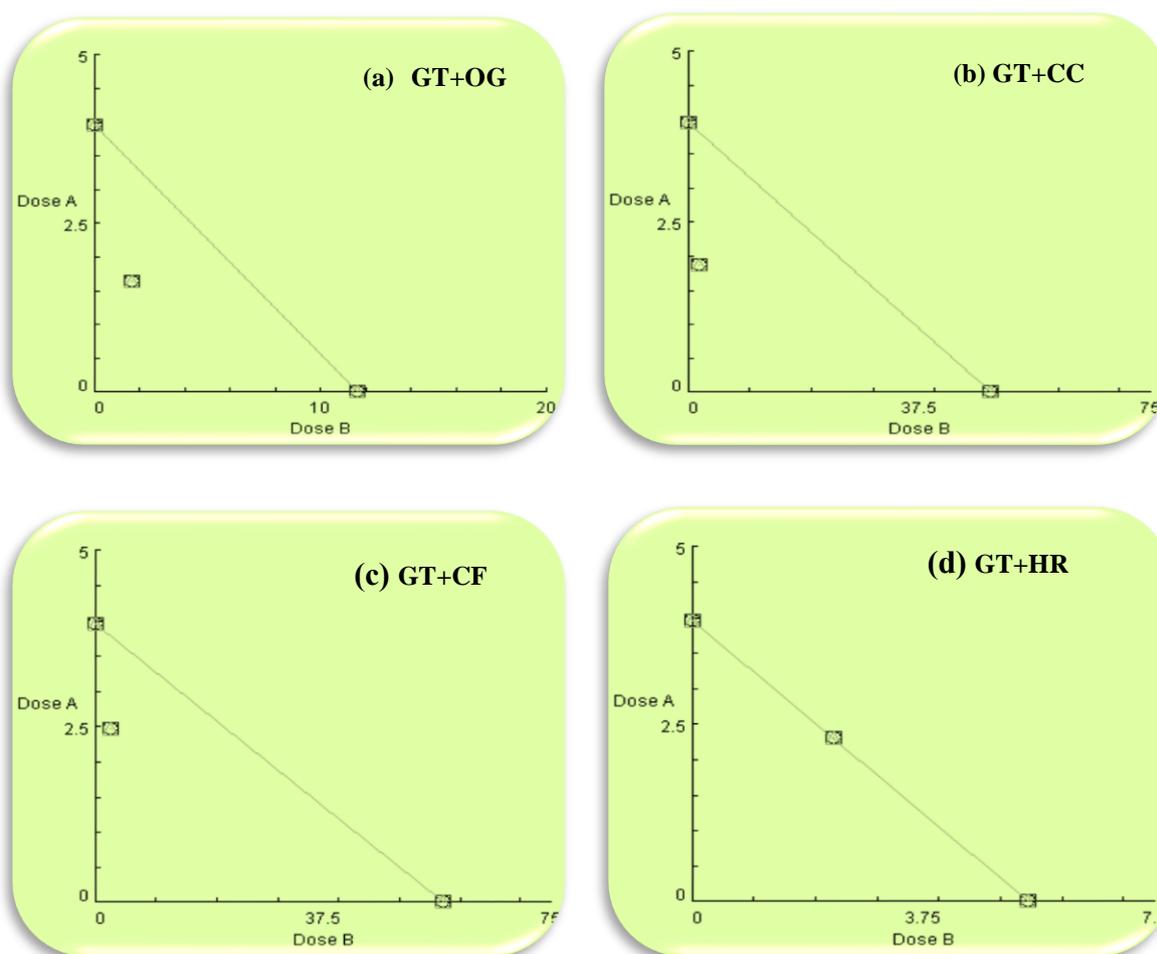


Fig. 23: Isobolograms showing interaction between (a) Green tea and *O. gratissimum* (GT+OG), (b) Green tea and *C. citratus* (GT+CC), (c) Green tea and *C. flexuosus* (GT+CF) and (d) Green tea and *H. rosa-sinensis* (GT+HR) at 1:1 ratio in ABTS assay. Dose A represents the EC₅₀ of GT in each graph and Dose B stands for EC₅₀ of OG or CC or CF or HR. The straight line (additive line) is made by dose A and B (EC₅₀ value). If the square symbol lies below the line of additivity it indicates synergistic interaction, above the line antagonistic and on this line, is the additive effect. The data are shown as MEAN \pm S.D for 3 independent experiments (each with three replicates of each test point).

In nitrite radical scavenging assay, all the samples displayed synergism with CI value in the following increasing order: GT+OG, GT+CC, GT+HR and GT+CF at EC₅₀, respectively as displayed in **Table 12**. The GT+OG combination displayed lower CI value ($p \leq 0.05$), and higher synergism compared to other combinations. In this assay, the isobolograms demonstrated that the EC₅₀ value lies below the line of additivity for all combinations that indicated synergistic interaction as depicted in **Fig. 24a-d**.

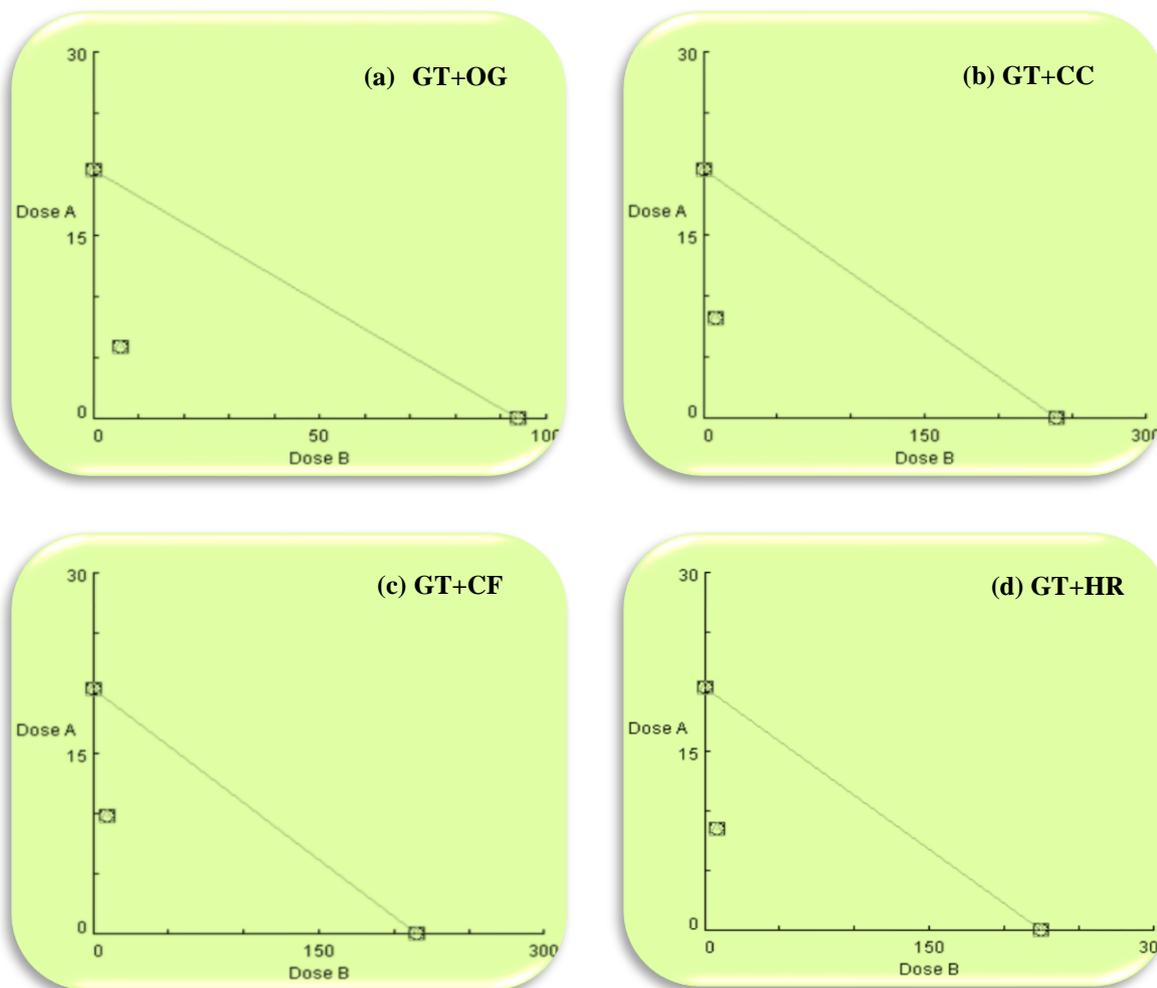


Fig. 24: Isobolograms showing interaction between (a) Green tea and *O. gratissimum* (GT+OG), (b) Green tea and *C. citratus* (GT+CC), (c) Green tea and *C. flexuosus* (GT+CF) and (d) Green tea and *H. rosa-sinensis* (GT+HR) at 1:1 ratio in NO assay. Dose A represents the EC₅₀ of GT in each graph and Dose B stands for EC₅₀ of OG or CC or CF or HR. The straight line (additive line) is made by dose A and B (EC₅₀ value). If the square symbol lies below the line of additivity it indicates synergistic interaction, above the line antagonistic and on this line, is the additive effect. The data are shown as MEAN \pm S.D for 3 independent experiments (each with three replicates of each test point).

In the LPO assay, GT+OG illustrated lower CI value ($p \leq 0.05$) than the rest of the combinations and indicates maximum synergism, followed by GT+CC, GT+HR and GT+CF, respectively as given in **Table 12**. In this assay, the EC_{50} value lies below the additivity line in all green tea combinations, thus demonstrating synergistic interactions as shown in **Fig. 25a-d**.

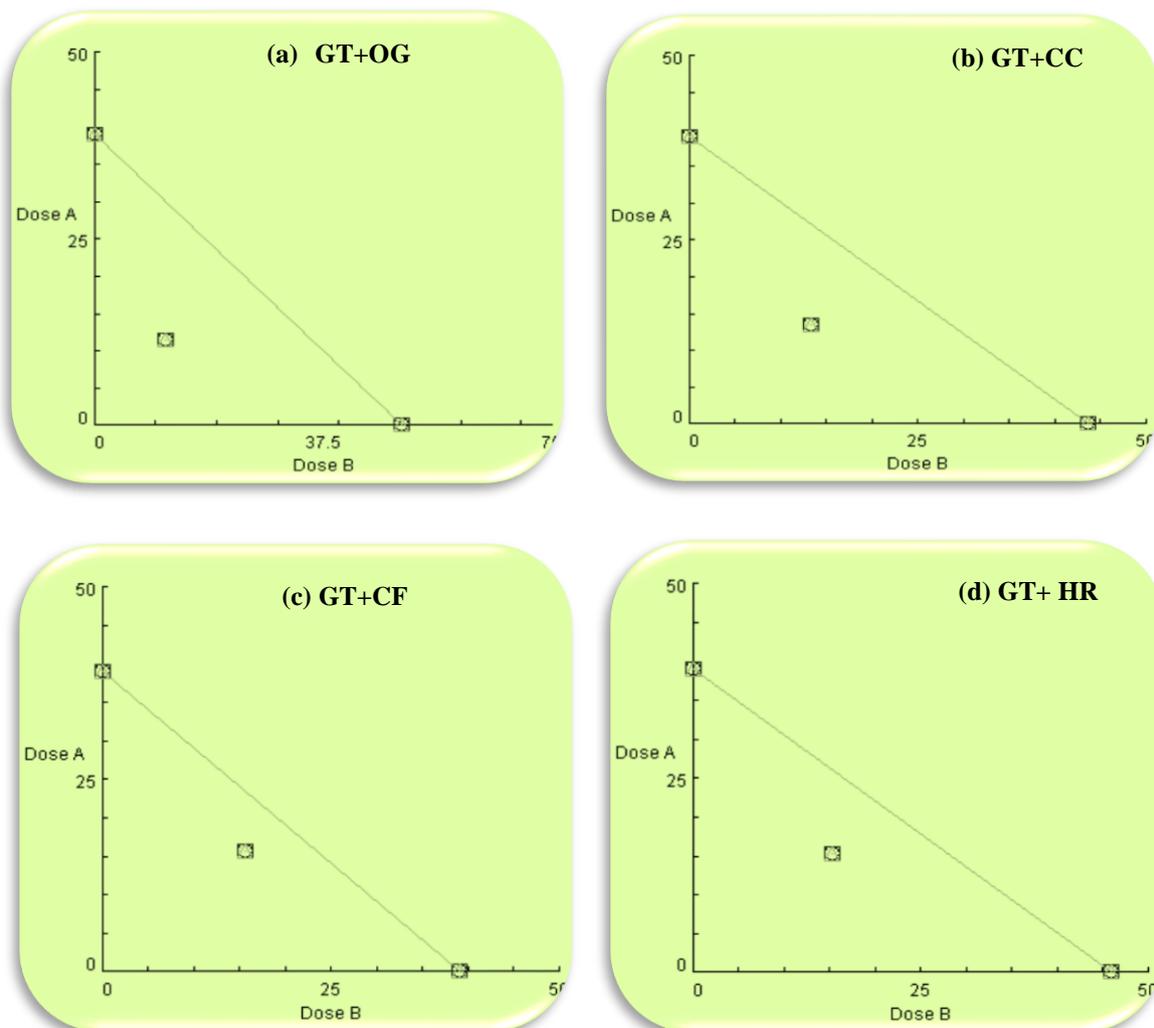


Fig. 25: Isobolograms showing interaction between (a) Green tea and *O. gratissimum* (GT+OG), (b) Green tea and *C. citratus* (GT+CC), (c) Green tea and *C. flexuosus* (GT+CF) and (d) Green tea and *H. rosa-sinensis* (GT+HR) at 1:1 ratio in LPO assay. Dose A represents the EC_{50} of GT in each graph and Dose B stands for EC_{50} of OG or CC or CF or HR. The straight line (additive line) is made by dose A and B (EC_{50} value). If the square symbol lies below the line of additivity it indicates synergistic interaction, above the line antagonistic and on this line, is the additive effect. The data are shown as $MEAN \pm S.D$ for 3 independent experiments (each with three replicates of each test point).

In anti-haemolysis assay, it was observed that CI value of different binary combinations are in following increasing order: GT+OG, GT+CC, GT+CF and GT+HR as given in **Table 13**. GT+OG, the only binary combination that demonstrated synergism in this assay, however the GT+CC, GT+CF and GT+HR combinations illustrated additive interactions. In GT+OG combination (**Fig.26a**), the EC₅₀ value lies below the additivity line that displayed synergism, while as in GT+CC, GT+CF and GT+HR, it lies near to the additivity line showed additive effect as given in **Fig. 26b,c and d**.

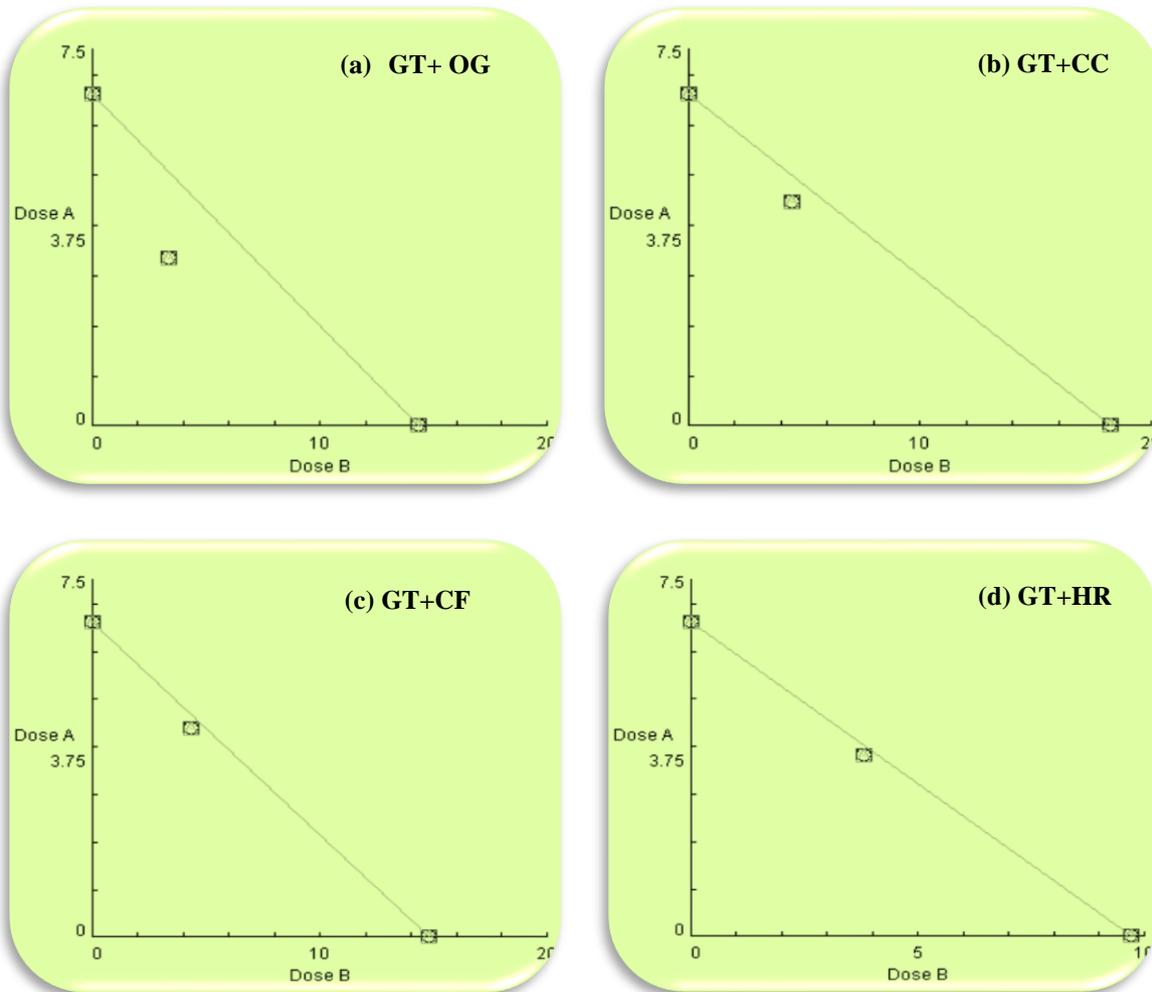
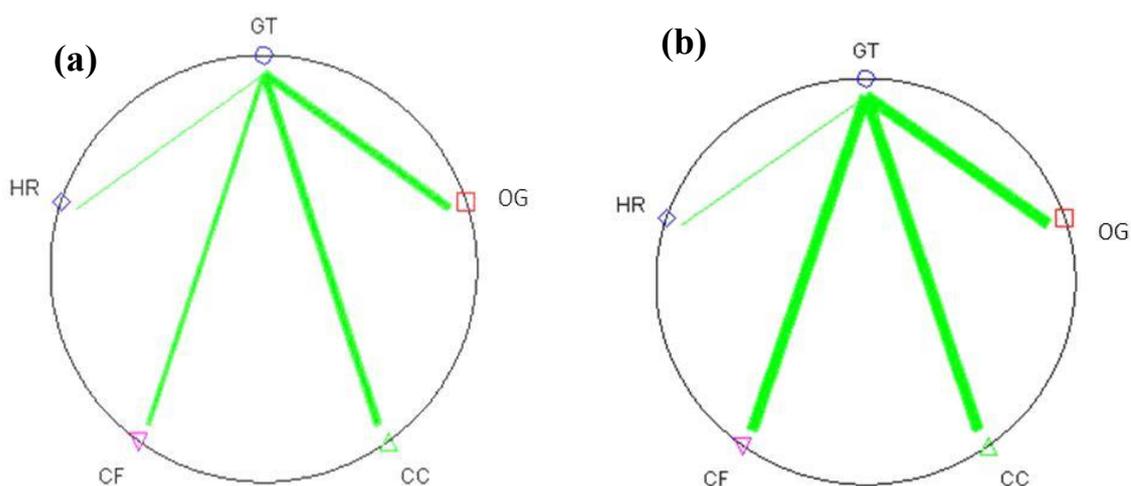


Fig. 26: Isobolograms showing interaction between (a) Green tea and *O. gratissimum* (GT+OG), (b) Green tea and *C. citratus* (GT+CC), (c) Green tea and *C. flexuosus* (GT+CF) and (d) Green tea and *H. rosa-sinensis* (GT+HR) at 1:1 ratio in haemolysis assay. Dose A represents the EC₅₀ of GT in each graph and Dose B stands for EC₅₀ of OG or CC or CF or HR. The straight line (additive line) is done by dose A and B (EC₅₀ value). If the square symbol lies below the line of additivity it indicates synergistic interaction, above the line antagonistic and on this line, is the additive effect. The data are shown as MEAN ± S.D for 3 independent experiments (each with three replicates of each test point).

6.2.2 Antioxidant interactions by Polygonogram

Polygonogram is the graphical representation that provides an easy representation of the overall results for interaction types between different drug combinations. The synergism is designated by solid lines and thin line presented the additive effect. The thickness of line connecting the two drugs/plant extracts display the strength of synergistic interaction.

In DPPH assay, the GT+OG and GT+CC demonstrated moderate synergism symbolized by thick solid line, GT+CF showed slight synergism represented by less thick solid line and GT+HR revealed nearly additive interaction symbolized by thin solid line as given in **Fig. 27a**. In the ABTS assay, GT+OG, GT+CC and GT+CF established synergistic interaction signified by more thick solid lines and GT+HR denoted by thin solid line revealed nearly additive interaction as shown in **Fig. 27b**. All the GT combinations indicated synergism in NO test represented by more thick solid lines as depicted in **Fig. 27c**. In LPO test, GT+OG and GT+CC showed synergism characterized by more thick solid line and GT+CF and GT+HR displayed moderate synergism shown by less thick lines as illustrated in **Fig. 27d**. GT+CC, GT+CF and GT+HR exhibited nearly additive interaction illustrated by thin solid lines and GT+OG combination demonstrated moderate synergism demonstrated by thick solid line as shown in **Fig.27e**.



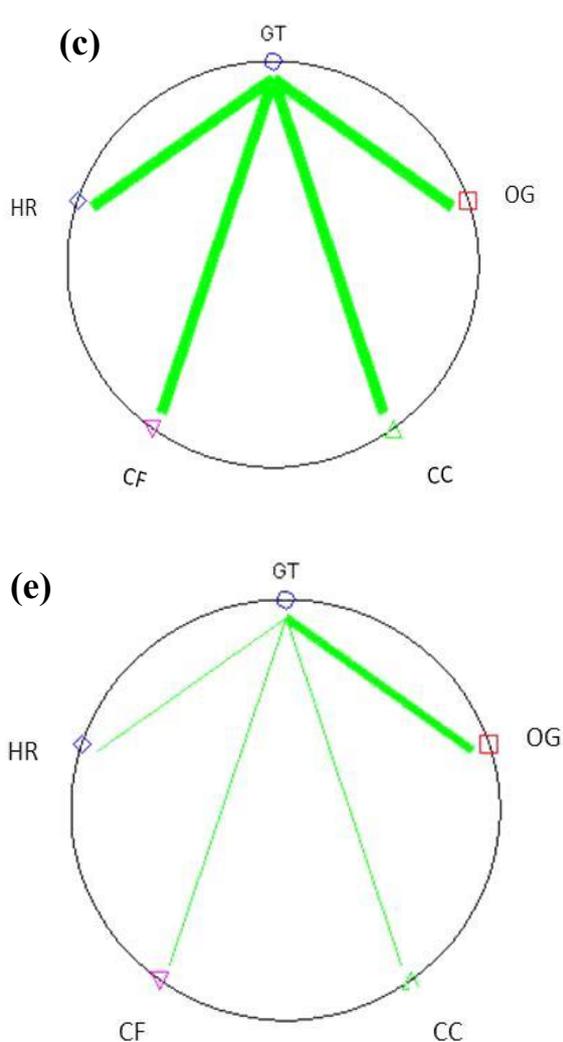


Fig. 27: Polygonogram demonstrating the antioxidant interactions between the green tea combinations (GT+OG, GT+CC, GT+CF and GT+HR at 1:1) for (a) DPPH (b) ABTS (c) NO (d) LPO (e) Haemolysis. The data are shown as MEAN \pm S.D for 3 independent experiments (each with three replicates of each test point). GT, OG, CF, CC and HR represent Green tea, *O. gratissimum*, *C. flexuosus*, *C. citratus* and *H. rosa -sinensis*. The 2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), lipid peroxidation (LPO) and nitric oxide (NO). Synergism is indicated by solid line, and thin line presented the additive effect. The thickness of line connecting the two drugs/plant extracts display the strength of synergistic interaction.

Table 12: Antioxidant capacity, combination index (CI) and type of antioxidant interaction between individual infusion and green tea (1:1) binary combination for various antioxidant assays.

| Assay | Infusion type | EC ₅₀ (µg/ml) | CI at EC ₅₀ | Type of interaction |
|-------------|---------------|-----------------------------|------------------------|---------------------|
| DPPH | GT | 12.92 ± 0.26 ^a | - | - |
| | OG | 53.59 ± 0.82 ^b | - | - |
| | CC | 295.27 ± 9.27 ^c | - | - |
| | CF | 155.37 ± 2.36 ^d | - | - |
| | HR | 38.82 ± 1.96 ^e | - | - |
| | GT+OG | 16.47 ± 0.27 ^a | 0.79 ^a | Moderate synergism |
| | GT+CC | 19.46 ± 0.85 ^a | 0.78 ^a | Moderate synergism |
| | GT+CF | 20.59 ± 1.06 ^a | 0.86 ^a | Slight synergism |
| | GT+HR | 18.44 ± 0.18 ^a | 0.95 ^b | Nearly additive |
| | ASC | 5.42 ± 0.34 ^f | - | - |
| ABTS | GT | 3.95 ± 0.11 ^a | - | - |
| | OG | 11.63 ± 0.36 ^b | - | - |
| | CC | 49.12 ± 1.10 ^c | - | - |
| | CF | 57.47 ± 2.51 ^d | - | - |
| | HR | 5.43 ± 0.41 ^{a,e} | - | - |
| | GT+OG | 3.28 ± 0.07 ^a | 0.55 ^a | Synergism |
| | GT+CC | 3.74 ± 0.005 ^{a,e} | 0.51 ^a | Synergism |
| | GT+CF | 4.93 ± 0.04 ^{a,e} | 0.66 ^b | Synergism |
| | GT+HR | 4.61 ± 0.28 ^{a,e} | 1.00 ^c | Nearly additive |
| | ASC | 6.11 ± 0.06 ^{a,e} | - | - |
| NO | GT | 20.42 ± 0.73 ^a | - | - |
| | OG | 94.70 ± 4.32 ^b | - | - |
| | CC | 267.28 ± 6.17 ^c | - | - |
| | CF | 214.93 ± 2.15 ^d | - | - |
| | HR | 225.18 ± 5.30 ^e | - | - |

| | | | | |
|-------------------|-------|-----------------------------|-------------------|--------------------|
| LPO | GT+OG | 11.89 ± 0.21 ^{a,f} | 0.35 ^a | Synergism |
| | GT+CC | 16.30 ± 0.54 ^a | 0.42 ^b | Synergism |
| | GT+CF | 19.58 ± 0.49 ^a | 0.52 ^c | Synergism |
| | GT+HR | 17.13 ± 0.47 ^a | 0.45 ^b | Synergism |
| | ASC | 6.18 ± 0.25 ^f | - | - |
| | GT | 39.03 ± 0.91 ^a | - | - |
| | OG | 50.19 ± 1.91 ^b | - | - |
| | CC | 43.62 ± 0.57 ^c | - | - |
| | CF | 39.13 ± 0.67 ^a | - | - |
| | HR | 45.96 ± 0.55 ^d | - | - |
| Haemolysis | GT+OG | 23.18 ± 0.59 ^e | 0.52 ^a | Synergism |
| | GT+CC | 26.83 ± 0.08 ^f | 0.65 ^b | Synergism |
| | GT+CF | 31.30 ± 0.40 ^g | 0.80 ^c | Moderate synergism |
| | GT+HR | 30.55 ± 0.34 ^g | 0.72 ^d | Moderate synergism |
| | BHT | 20.32 ± 0.70 ^h | - | - |
| | GT | 6.62 ± 0.17 ^a | - | - |
| | OG | 14.33 ± 0.17 ^b | - | - |
| | CC | 18.27 ± 0.46 ^c | - | - |
| | CF | 14.76 ± 0.15 ^b | - | - |
| | HR | 9.71 ± 0.27 ^d | - | - |
| | GT+OG | 6.69 ± 0.11 ^a | 0.73 ^a | Moderate synergism |
| | GT+CC | 8.94 ± 0.13 ^e | 0.92 ^b | Nearly additive |
| | GT+CF | 8.76 ± 0.18 ^e | 0.95 ^b | Nearly additive |
| | GT+HR | 7.63 ± 0.11 ^f | 0.96 ^b | Nearly additive |
| | ASC | 7.82 ± 0.15 ^f | - | - |

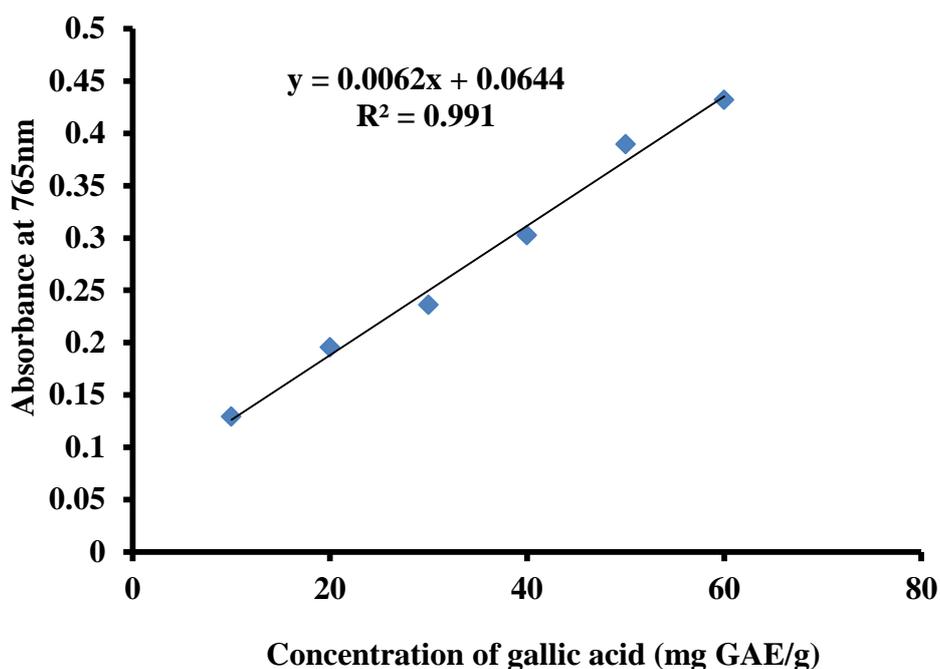
GT, OG, CC, CF and HR represent Green tea, *O. gratissimum*, *C. citratus*, *C. flexuosus* and *H. rosa-sinensis*. ASC (ascorbic acid), BHT (butylated hydroxytoluene), EC₅₀ (Effective concentration causing 50% scavenging activity); CI (Combination index). The data are shown as MEAN ± S.D for 3 independent tests (each with two replicates of each test point). The range of CI values and the type of interaction was followed as given by Chou (2006) are: < 0.1 (very strong synergism), 0.1-0.3 (strong synergism), 0.3-0.7 (synergism), 0.7-0.85 (moderate synergism), 0.85- 0.90 (slight synergism), 0.90-1.10 (nearly additive), 1.10-1.20 (slightly antagonism), 1.20-1.45 (moderate antagonism), 1.45-3.3 (antagonism), 3.3-10 (strong antagonism) and > 10 (very strong antagonism). Different alphabets depicted a large difference at $p \leq 0.05$ in the same column for a particular

assay type. 2, 2-diphenyl-1-picrylhydrazyl (DPPH), [(2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] (ABTS), nitric oxide (NO) and lipid peroxidation (LPO).

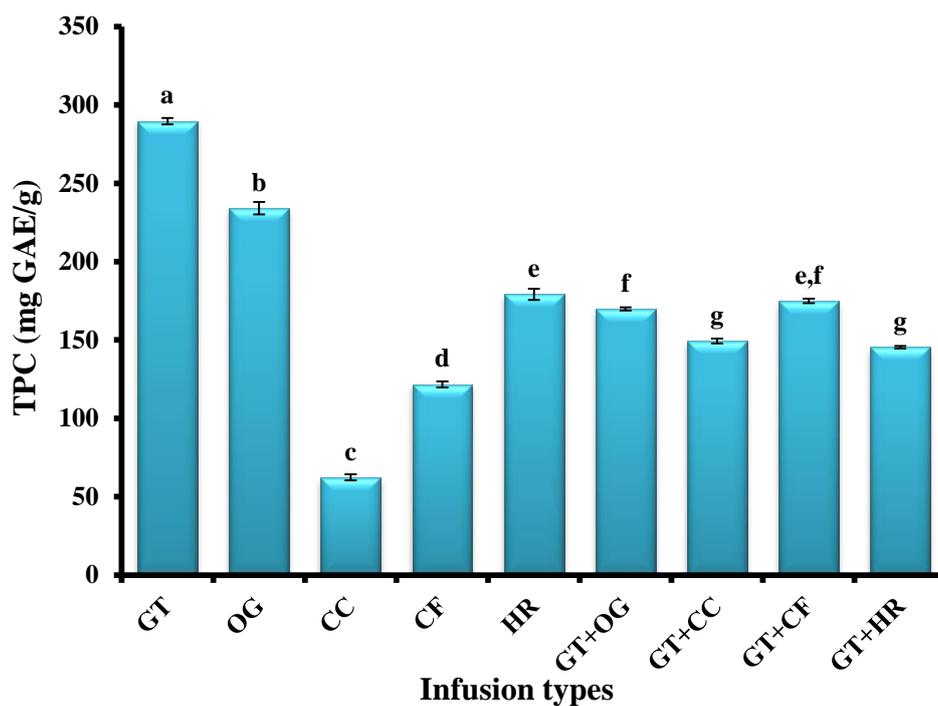
6.2.3 Total phenolic content and Flavonoid content of different infusions

6.2.3.1 Total phenolic content of green tea and medicinal plants alone and in combinations

The therapeutic and preventive effects of various medicinal plants or herbs have been ascribed to the presence of polyphenolic complexes. Total phenolic content (TPC) as measured on the basis of the standard curve of gallic acid depicted in **Fig. 28a**. It was noticed that the highest TPC was demonstrated by GT followed by OG, HR, GT+CF, GT+OG, GT+CC, GT+HR, CF and lowest in CC, respectively as given in **Fig. 28b**. GT possesses highest total phenolic content than other infusions ($p \leq 0.05$), while CC exhibited lowest TPC among all the infusions studied as given in **Table 13**.



(a)

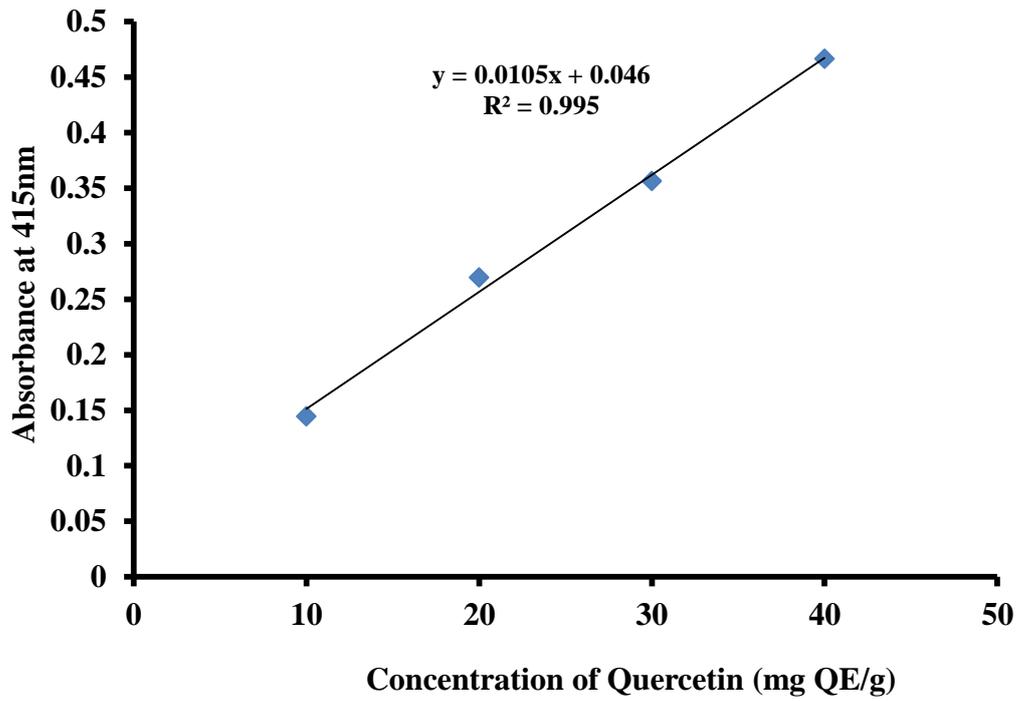


(b)

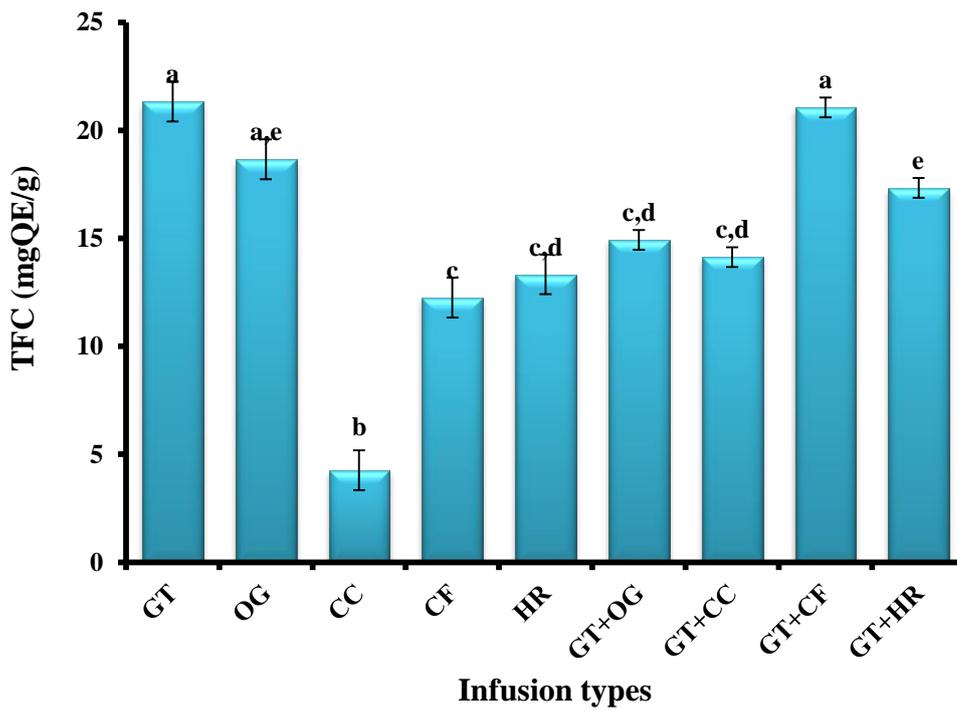
Fig. 28: (a) The standard calibration curve of gallic acid and (b) Total phenolic content (TPC) of different infusions alone and in combination. The data are shown as MEAN \pm S.D for 3 independent tests (each with three replicates of each test point). Different alphabets depicted significant difference at $p \leq 0.05$ among the TPC of different infusions. GT, OG, CF, CC and HR represent Green tea, *O. gratissimum*, *C. flexuosus*, *C. citratus* and *H. rosa-sinensis*.

6.2.3.2 Total flavonoid content of green tea and medicinal plants alone and in combinations

The total flavonoid content (TFC) was calculated from quercetin standard curve as displayed in **Fig. 29a**. The TFC of various infusions are given in the following decreasing order: GT, GT+CF, OG, GT+HR, GT+OG, GT+CC, HR, CF and CC, respectively as depicted in **Fig. 29b**. GT, GT+CF and OG demonstrated similar ($p \geq 0.05$) but higher total flavonoid content as compared to other infusions ($p \leq 0.05$), while as CC showed lowest TFC as shown in **Table 13**.



(a)



(b)

Fig. 29: (a) The standard calibration curve of quercetin and (b) Total flavonoid content (TFC) of different infusions alone and in combination. The data are shown as MEAN \pm S.D for 3 independent tests (each with three replicates of each test point). Different alphabets depicted significant difference at $p \leq 0.05$ among the TFC of different

infusions. GT, OG, CF, CC and HR represent Green tea, *O. gratissimum*, *C. flexuosus*, *C. citratus* and *H. rosa-sinensis*.

6.2.4 Comparison of theoretical and experimental value of total phenolic or flavonoid content

Theoretical value of TPC/TFC is average value of the individual infusions of a binary mixture. TPC/TFC experimental value was found to be significantly lower than the theoretical value for all binary mixtures except a minor increase in experimental value was observed for GT+CF whereas no difference was detected in flavonoid content of GT+CC as depicted in **Table 13**.

Table 13: Total phenolic and flavonoid content of different infusions alone and in combination

| Samples | Theoretical value TPC (mg GAE/g) | Experimental value TPC (mgGAE/g) | Theoretical value TFC (mg QE/g) | Experimental value TFC (mg QE/g) |
|---------|----------------------------------|----------------------------------|---------------------------------|----------------------------------|
| GT | - | 289.63 ± 1.97 ^a | - | 21.53 ± 1.07 ^a |
| OG | - | 234.15 ± 3.94 ^b | - | 19.50 ± 0.45 ^{a,e} |
| CC | - | 62.32 ± 1.93 ^c | - | 4.87 ± 1.06 ^b |
| CF | - | 121.67 ± 1.93 ^d | - | 12.80 ± 0.84 ^c |
| HR | - | 179.09 ± 3.59 ^e | - | 14.32 ± 0.30 ^{c,d} |
| GT+OG | 261.89 ± 1.34* | 169.87 ± 0.96 ^f | 20.52 ± 0.48* | 15.18 ± 0.43 ^{c,d} |
| GT+CC | 175.97 ± 0.81* | 149.33 ± 1.52 ^g | 13.71 ± 0.53 | 14.67 ± 0.70 ^{c,d} |
| GT+CF | 205.65 ± 0.18* | 174.92 ± 1.34 ^{e,f} | 17.67 ± 0.42* | 21.18 ± 1.06 ^a |
| GT+HR | 234.36 ± 2.42* | 145.46 ± 0.81 ^g | 18.43 ± 0.15 | 17.54 ± 0.80 ^e |

GT, OG, CF, CC and HR represent Green tea, *O. gratissimum*, *C. flexuosus*, *C. citratus* and *H. rosa-sinensis*. TPC (total phenolic content). Results are stated as MEAN ± S.D, n = 3. The different alphabets in the same column designate the significant difference ($p \leq 0.05$) among different infusions. * indicated significant difference ($p \leq 0.05$) between theoretical value (average value of the individual infusions) and experimental value of total phenolic content (TPC) and total flavonoid content (TFC) of a green tea combinations.

6.2.5 Correlation study between total phenolic or flavonoid content and antioxidant activity

A correlation analysis between the TPC/TFC and EC₅₀ (antioxidant activity) of individual infusions and binary combinations was performed separately and categorized according to

Hinkle et al., 2003. In case of individual infusions very high to high negative correlation was observed for various antioxidant tests with the correlation coefficient (r) as follows: TPC/TFC-DPPH scavenging activity (r = -0.912/-0.954), TPC/TFC-ABTS scavenging activity (r = -0.839/-0.847), TPC/TFC-NO scavenging activity (r = -0.938/-0.856), and TPC/TFC-anti haemolytic activity (r = -0.822/-0.928), and negligible correlation was noticed between the TPC/TFC and LPO (r = 0.065/-0.043). A negligible association was demonstrated between TPC and various antioxidant activities DPPH (r= 0.024), ABTS (r = 0.021), NO (r= 0.046), LPO (r= -0.139) and Anti-haemolytic (r= -0.134) for GT combinations (GT+OG, GT+CC, GT+CF and GT+HR) but in case of flavonoid content negligible to high positive correlation was observed: TFC-anti haemolytic activity (r = 0.109), DPPH (r = 0.435), TFC-NO (r = 0.571), TFC-LPO (r = 0.612) and TFC-ABTS (r = 0.742) as shown in **Table 14**.

Table 14: A correlation analysis between the antioxidant capacity and total phenolic/ flavonoid content of individual infusions and green tea combinations.

| Antioxidant assays | TPC/TFC of all infusions | Correlation coefficient (r) of individual samples | Type of correlation | Correlation coefficient (r) of GT combinations | Type of correlation |
|--------------------|--------------------------|---|---------------------|--|---------------------|
| DPPH | TPC | -0.912 | Very high(-ve) | 0.024 | Negligible |
| | TFC | -0.954 | Very high(-ve) | 0.435 | Low (+ve) |
| ABTS | TPC | -0.839 | High (-ve) | 0.021 | Negligible |
| | TFC | -0.847 | High (-ve) | 0.742 | High (+ve) |
| NO | TPC | -0.938 | Very high(-ve) | 0.046 | Negligible |
| | TFC | -0.856 | High (-ve) | 0.571 | Moderate (+ve) |
| LPO | TPC | 0.065 | Negligible | -0.139 | Negligible |
| | TFC | -0.043 | Negligible | 0.612 | Moderate (+ve) |
| Haemolysis | TPC | -0.822 | High(-ve) | -0.134 | Negligible |
| | TFC | -0.928 | Very high(-ve) | 0.109 | Negligible |

The 2, 2-diphenyl-1-picrylhydrazyl (DPPH), [(2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] (ABTS), nitric oxide (NO), lipid peroxidation (LPO), Total phenolic content (TPC), Total flavonoid content (TFC), +ve (Positive correlation) and -ve (negative correlation). 0.90 to 1.00/ -0.90to -1.00: very high positive/

very high negative correlation; 0.70 to 0.90 (-0.70 to -0.90) high positive (negative) correlation; 0.50 to 0.70 (-0.50 to -0.70) moderate positive (negative) correlation; 0.30 to 0.50 (-0.30 to -0.50) low positive (negative) correlation and 0.00 to 0.30 (0.00 to -0.30) negligible correlation (Hinkle et al., 2003).

6.3 FTIR analysis of individual infusions and binary combinations

The FTIR analysis of green tea (GT), *O. gratissimum* (OG), *C. citratus* (CC), *C. flexuosus* (CF) and *H. rosa-sinensis* (HR) were evaluated. The powdered samples of all extracts were loaded in ATR-FTIR spectra with a scan range from 600-4000 cm^{-1} in the mid IR region. The aqueous extracts of these samples are complex mixture of phytochemicals. Their FTIR spectra demonstrated an overlap of absorption peaks of functional groups absorbing belonging similar wavelength.

6.3.1 Green tea (GT)

The FTIR spectrophotometric analysis showed the presence of different functional groups in green tea dried powder as shown in **Fig. 30**. Green tea (GT) spectra demonstrated a broad (3000 – 3600 cm^{-1}) and intense peak centered at 3269.45 cm^{-1} corresponding to overlapping of the hydroxyl group of alcohols or phenols, CH stretch of alkene, arenes and NH stretch of amine groups. The band at 2928.04 cm^{-1} , exhibited OH stretching of carboxylic acid and C-H stretching of alkanes. The band obtained at 1572.04 cm^{-1} demonstrated N-H bending of amide and C=C stretching aromatic groups. Absorption peak at 1492.95 cm^{-1} revealed the C=C stretching of aromatic compounds. The 1384.94 cm^{-1} peak demonstrated the presence of C-H bending of alkanes. The bands 1265.35 cm^{-1} and 1203.62 cm^{-1} may be attributed to overlapping of C-N stretching of primary and secondary amines and C-O stretching carboxylic acids. The peak 1041.60 cm^{-1} assigned the CO stretching of alcohols/phenol/ester and CN stretching of amine. A band at 929.72 cm^{-1} demonstrated = C-H bending of alkenes. However, 810.13 cm^{-1} and 871.85 cm^{-1} peaks may attributed the presence of = CH bending of alkenes and CH bending of aromatic compounds. Aromatic compounds also have a set of small absorption bands between 1600-2000 cm^{-1} called the overtones as depicted in **Table 15**.

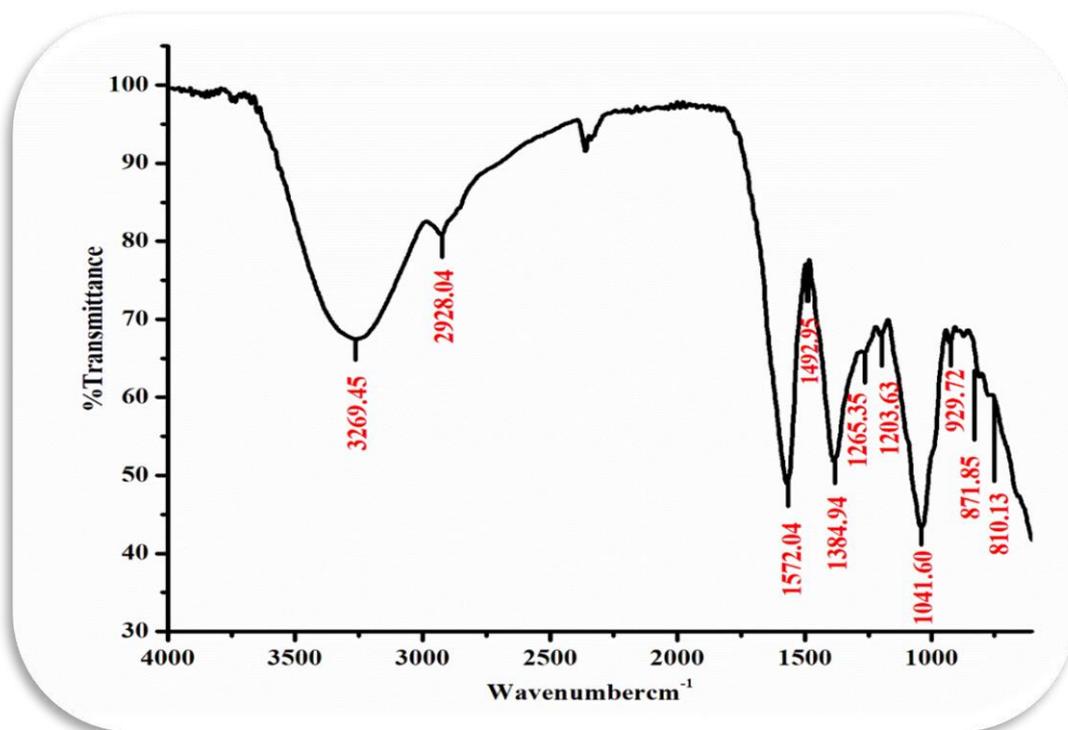


Fig. 30: FTIR spectra of green tea with a scan range from 600-4000cm⁻¹

6.3.2 *Ocimum gratissimum* (OG)

FTIR spectrum of *O. gratissimum* depicted in **Fig. 31** displayed a broad band at 3271.38 cm⁻¹ corresponding the overlapping of hydroxyl group of alcohols or phenols, CH stretch of alkene, arenes and NH stretch of amine groups. The peaks at 2931.90 cm⁻¹ exhibited the O-H stretching of carboxylic acids and CH stretching of alkane, 1568.18 cm⁻¹ (NH bending of amide and C=C stretching of aromatic groups), 1377.22cm⁻¹ (CH bending of alkanes), 1261.49 cm⁻¹ (CO stretching carboxylic acids), 1199.76 cm⁻¹ (CN stretching of amine), 1031.95 cm⁻¹ (CO stretching of alcohols/phenol/ester, CN stretching of amine), 920.08 cm⁻¹ (=C-H bending of alkenes), 812.06 cm⁻¹ (=C-H bending of alkenes and C-H bending of aromatic compounds) and 765.77 cm⁻¹ (C-Cl stretch of alkyl halides) as given in **Table 15**.

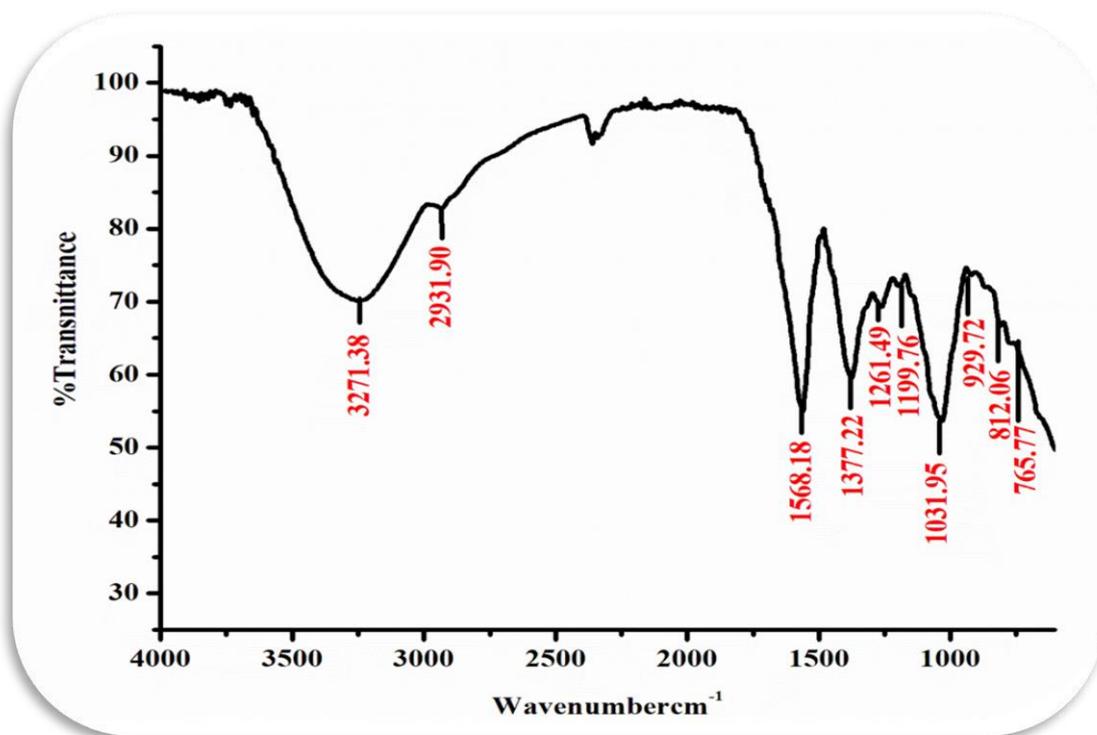


Fig. 31: FTIR spectra of *O. gratissimum* (OG) with a scan range from 600-4000cm⁻¹

6.3.3 *Cymbopogon citratus* (CC)

The FTIR spectrum of *C. citratus* given in **Fig. 32**, demonstrated broad band at 3273.31 cm⁻¹ corresponding to overlapping of hydroxyl group of alcohols or phenols, CH stretch of alkene, arenes and NH stretch of amine groups. The bands at 2920.32 cm⁻¹ may be due to the CH stretching of alkanes and OH stretching of carboxylic acids, 1689.70 cm⁻¹ (C=O stretching of carboxylic acids), 1604.83 cm⁻¹ (NH bending of amide groups), 1516.10 cm⁻¹ (C=C vibrations of aromatic structures), 1448.59 cm⁻¹ (CH bending of alkane and C=C stretching of aromatic compounds), 1363.72 cm⁻¹ (CH bending of alkanes), 1205.55 cm⁻¹ (C-N stretching of amine and CO stretching of ether), 1139.97 cm⁻¹ (CO stretch of alcohols and CN stretching of amine), 1035.81cm⁻¹ (CO stretching of phenol/ alcohols/ether and ester), 875.71 cm⁻¹ (=C-H bending of alkenes and CH bending of aromatic compounds) and 603.74 cm⁻¹ (C-Cl stretch of alkyl halides) as displayed in **Table 15**.

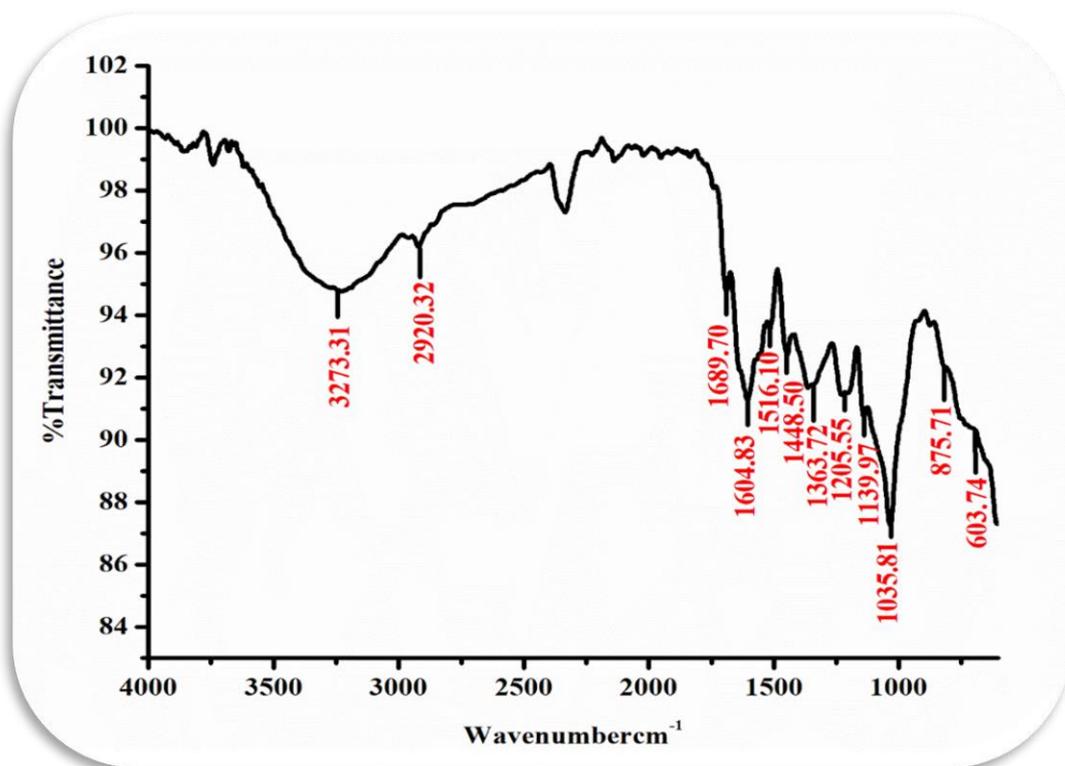


Fig. 32: FTIR spectra of *C.citratus* (CC) with a scan range from 600-4000cm⁻¹

6.3.4 *Cymbopogon flexuosus* (CF)

The FTIR spectra of *C. flexuosus* illustrated in **Fig. 33** exhibited broad band at 3257.88 cm⁻¹ corresponding the overlapping of hydroxyl group of alcohols or phenols, CH stretch of alkene, arenes and NH stretch of amine groups. The peaks at 2922.25 cm⁻¹ exhibited the O-H stretching of carboxylic acids and CH stretching of alkanes, 1570.11 cm⁻¹ (NH bending of amide and C=C vibrations of aromatic structures), 1383.01 cm⁻¹ (C-H bending of alkanes), 1267.27 cm⁻¹ (CO stretching of carboxylic acids), 1197.76 cm⁻¹ (CO stretching of alcohols/phenols and CN stretching of amine), 1035.81 cm⁻¹ (CO stretching of phenol/alcohols/ether), 923.93 cm⁻¹ (=C-H bending of alkenes), 873.78 and 810.13 cm⁻¹ (= CH bending of alkenes and CH bending of aromatic compounds) and 667.39 cm⁻¹ (C-Cl stretching of alkyl halide) as shown **Table 15**.

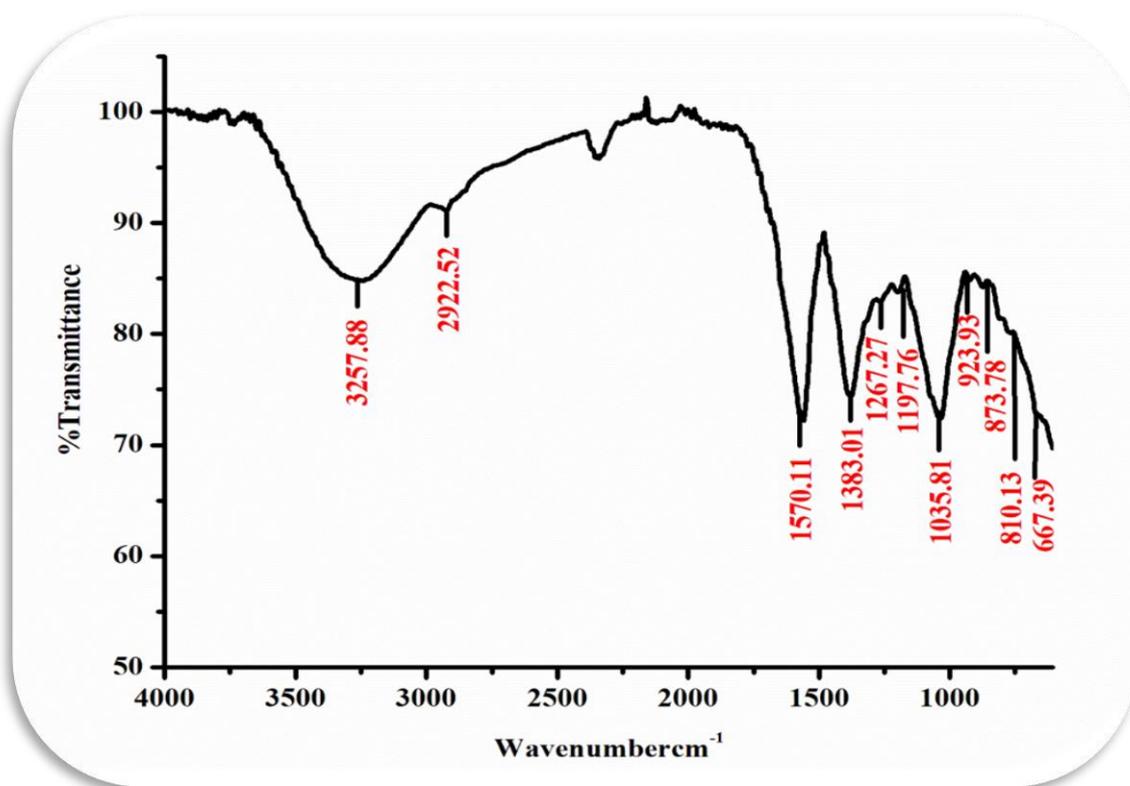


Fig. 33: FTIR spectra of *C. flexuosus* (CF) with a scan range from 600-4000 cm^{-1}

6.3.5 *Hibiscus rosa-sinensis* (HR)

The FTIR spectra of *H. rosa sinensis* as displayed in **Fig. 34** displayed a wide peak at 3282.95 cm^{-1} corresponding the overlapping of hydroxyl group of alcohols or phenols, C-H stretch of alkene, arenes and NH stretch of amine groups. The peaks at 2924.18 cm^{-1} , exhibited the C-H stretch of alkane and O-H stretch of carboxylic acids, 1564.32 cm^{-1} (NH bending of amide and C=C vibrations of aromatic structures), 1379.15 cm^{-1} (CH bending of alkanes), 1265.35 cm^{-1} (CO stretching carboxylic acids), 1199.76 cm^{-1} (CO stretching of alcohols/phenols and CN stretching of amine), 1037.74 cm^{-1} (CO stretching of phenol/alcohols/ether), 923.93 cm^{-1} (=CH bending of alkenes), 875.71 and 808.20 cm^{-1} (=CH bending of alkenes and C-H bending of aromatic compounds) and 661.61 cm^{-1} (C-Cl of alkyl halides) as illustrated in **Table 15**.

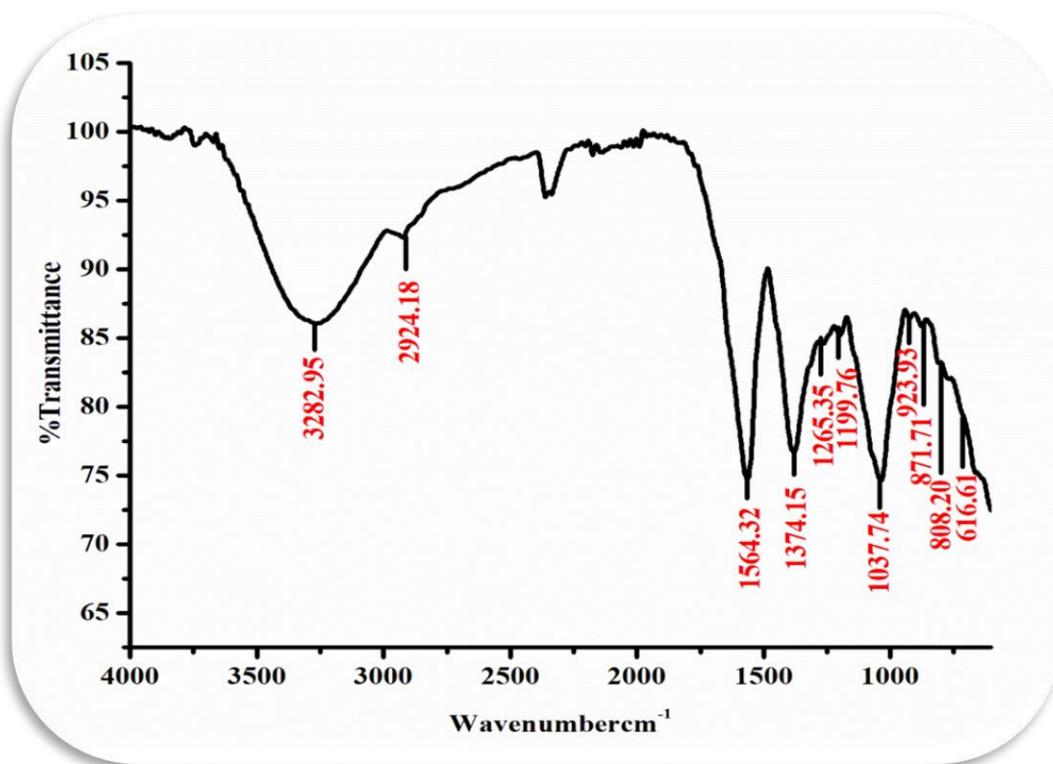


Fig. 34: FTIR spectra of *H.rosa sinensis* (HR) with a scan range from 600-4000cm⁻¹

Table 15: The absorption spectrum of aqueous extracts of green tea, *O. gratissimum*, *C. citratus*, *C. citratus* leaves and *H. rosa – sinensis* flower.

| Functional groups | Absorption spectrum, frequency cm ⁻¹ | | | | | |
|-------------------|---|------------------------|--|---|--|--|
| | Component (Peaks) | Green tea extract (GT) | <i>Ocimum gratissimum</i> extract (OG) | <i>Cymbopogon citratus</i> extract (CC) | <i>Cymbopogon flexuosus</i> Extract (CF) | <i>Hibiscus rosa – sinensis</i> extract (HR) |
| | Wave number (cm ⁻¹) | | | | | |
| Alcohol/Phenol | O-H (Stretch , H-bounded) (3200-3600) | 3269.45 | 3271.38 | 3273.31 | 3257.88 | 3282.95 |
| | C-O (Stretch) of alcohol (970-1250) | 1041.60 | 1031.95, 1199.76 | 1035.81, 1139.97 | 1035.81, 1197.76 | 1037.74, 1199.76 |
| Carboxylic acids | C=O (Stretch) (1680-1710) | - | - | 1689.70 | - | - |
| | O-H (Stretch) (2500-3500) | 2928.04 | 2931.90, | 2920.32, | 2922.25 | 2924.18 |
| | C-O (Stretch) (1210-1320) | 1265.35 | 1261.49 | - | 1267.27 | 1265.35 |
| Alkane | C-H (Stretch) (2850-3000) | 2928.04 | 2931.90 | 2920.32 | 2922.25 | 2924.18 |
| | C-H (Bending) (1350-1480) | 1384.94 | 1377.22 | 1363.72, 1448.59 | 1383.01 | 1379.15 |
| Amine | N-H (Stretch) (2280-3380) | 3269.45 | 3271.38 | 3273.31 | 3257.88 | 3282.95 |
| | C-N (stretch) (1000-1250) | 1041.60, 1203.62 | 1199.76 | 1139.97, 1205.55 | 1197.76 | 1199.76, 1265.35 |
| Amide | N-H (Bending) (1550-1640) | 1572.04 | 1568.58 | 1604.83 | 1570.11 | 1564.32 |
| Alkenes | =C-H (Bending) (675-1000) | 810.13, 871.85, 929.72 | 765.77, 812.06, 920.08 | 875.71 | 810.13, 873.78, 923.93 | 808.20, 875.71, 923.93 |
| Ether | C-O (Stretch) (1000-1300) | | 1031.95 | 1035.81 | 1035.81 | 1037.74 |
| Ester | C-O (Stretch) (1000-1300) | 1041.60 | 1031.95 | 1035.81, 1205.55 | - | - |
| Aromatic | C=C (Stretch) (1400-1600) | 1492.95, 1572.04 | 1568.18 | 1448.59, 1516.10 | 1570.11 | 1564.32 |
| | C-H (Bend) (680-900) | 810.13, 871.85 | 812.06 | 875.71 | 810.13, 873.78 | 808.20, 875.71 |
| Alkyl halide | C-Cl (Stretch) (600-800) | - | 765.77 | 603.74 | 667.39 | 661.61 |

GT, OG, CF, CC and HR represent Green tea, *O. gratissimum*, *C. flexuosus*, *C. citratus* and *H. rosa-sinensis*.

6.3.6 Comparison of FTIR spectra of individual samples

It can be clearly depicted from **Fig. 35** that GT demonstrated maximum peak intensity in the region between 3000-3600 cm^{-1} , followed by OG, CF, HR and least intensity of CC infusion. The spectral pattern of all infusions was found to be similar, but the differences were observed in intensities and width of peaks and a minor shift in wavenumbers. The sharp absorption bands were also observed in the region between 1500-1750, 1500-1250 and 1000-1200 cm^{-1} in all the samples except CC. GT and OG showed similar peak intensity whereas CF and HR samples demonstrated comparable peak intensity in 3000-3600 cm^{-1} region, respectively.

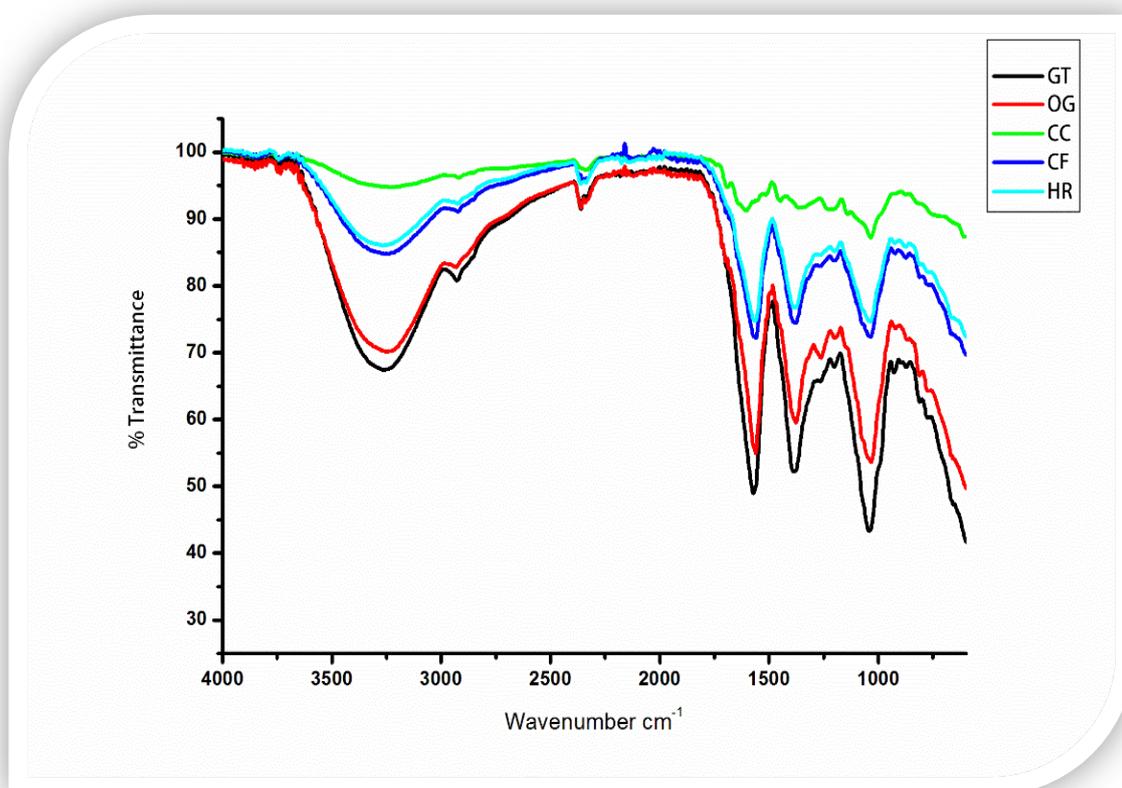


Fig. 35: Comparison of FTIR spectra of green tea (GT), *O. gratissimum* (OG), *C. citratus* (CC), *C. flexuosus* (CF) and *H. rosa-sinensis* (HR) samples.

6.3.7 Correlation between FTIR peak intensity and total phenolic content/antioxidant activity

The peak intensity in the region 3000-3600 cm^{-1} derived from the ATR-FTIR spectrum of GT, OG, CC, CF and HR were selected for correlation study. A correlation study was

conducted between intensity of absorbance peak ($3257.88\text{-}3282.95\text{cm}^{-1}$) and TPC or antioxidant activity. There were noticed very high negative correlation between absorbance and TPC ($r = - 0.950$), NO ($r = 0.975$) and high positive correlation in DPPH ($r = 0.802$), whereas a moderate positive correlation for haemolysis ($r = 0.639$) and ABTS ($r = 0.664$), and negligible correlation in case of LPO ($r = - 0.059$) as shown in **Table 16**.

Table 16: A correlation analysis between intensity of absorbance peak ($3258\text{-}3283\text{cm}^{-1}$) and total phenolic content or antioxidant activity of individual infusions.

| Parameter | Correlation coefficient (r) | Type of correlation |
|------------|-----------------------------|---------------------|
| TPC | -0.950 | Very high (-ve) |
| DPPH | 0.802 | High (+ve) |
| ABTS | 0.664 | Moderate (+ve) |
| NO | 0.975 | Very high (+ve) |
| LPO | - 0.059 | Negligible |
| Haemolysis | 0.639 | Moderate (+ve) |

The 2, 2-diphenyl-1-picrylhydrazyl (DPPH), [(2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] (ABTS), nitric oxide (NO), lipid peroxidation (LPO), Total phenolic content (TPC), positive correlation(+ve) and Fourier transform infrared spectrum (FTIR).

6.4. FTIR analysis of green tea combinations

6.4.1 Green tea and *O. gratissimum* combination (GT+OG)

The broad and intense band of GT+OG binary combination obtained between the region of $3000\text{-}3600\text{ cm}^{-1}$ gives a wide and strong peak at 3257.88 cm^{-1} indicated the overlapping of hydroxyl group of alcohols or phenols, CH stretch of alkene, arenes and N-H stretch of amine groups. The absorption bands at 2933.83 may assigned CH/OH stretch of alkanes and carboxylic acids, 1602.90 cm^{-1} (NH bending of amide groups), 1442.80 cm^{-1} (C=C stretching of aromatic groups and CH bending of alkanes), 1352.14 cm^{-1} (CH bending of alkanes and C-N stretching of amine groups), 1217.12 cm^{-1} (CO stretching carboxylic acids and CN stretching of amines) and 1045.45 cm^{-1} (CO stretching of phenol/alcohol/ester and ether) shown in **Fig. 36** and **Table 17**.

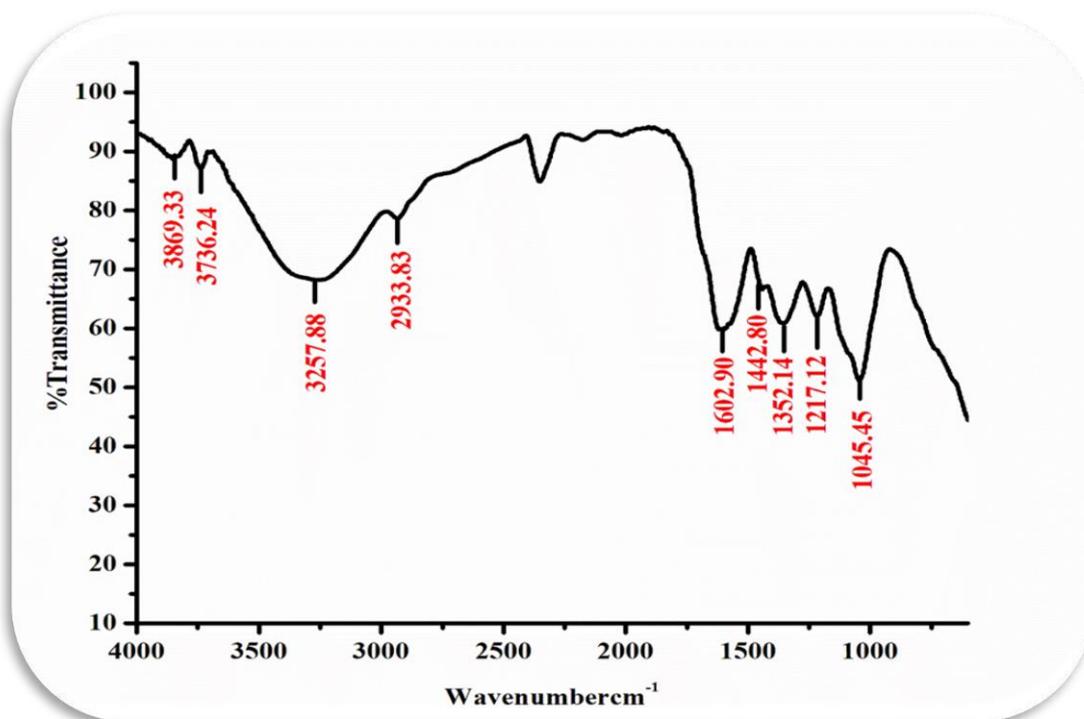


Fig. 36: FTIR spectra of green tea and *O.gratissimum* combination with a scan range from 600-4000cm⁻¹

6.4.2. Green tea and *C. citratus* combination (GT+CC)

The FTIR spectra of GT+CC binary combination as given in **Fig. 37**, exhibited a peak 3730.45 cm⁻¹ that assigned (OH stretch). The broad band in the region between 3000-3600 cm⁻¹ gives a band at 3205.80 cm⁻¹ showed the overlapping of hydroxyl group of alcohols or phenols, the CH stretch of alkene, arenes and NH stretch of amine groups. The absorption bands at 2933.83 may be assigned CH/ OH stretching of alkanes/carboxylic acids, 1693.56 cm⁻¹ (C=O stretching of carboxylic acids), 1599.04 cm⁻¹ (NH bending of amide and C=C aromatic stretch), 1516.10 cm⁻¹ (C=C stretching of aromatic groups), 1452.45 cm⁻¹ (C=C stretching of aromatic groups and CH bending of alkanes), 1369.50 cm⁻¹ (CH bending of alkanes), 1238.34 cm⁻¹ (CO stretch of carboxylic acids and CN stretch of amines), 1145.75 cm⁻¹ (CN stretching of amine), 1037.74 cm⁻¹ (CO stretching of alcohols/phenols/ester/ether), 929.72 cm⁻¹ (=C-H bending of alkenes), 815.92 cm⁻¹ (= CH bending of alkenes and C-H bending of aromatic compounds), 763.84 cm⁻¹ (CH bend of aromatic group and C-Cl stretch of alkyl halides) and 671.25 cm⁻¹ (C-Cl stretching of alkyl halides) as displayed in **Table 17**.

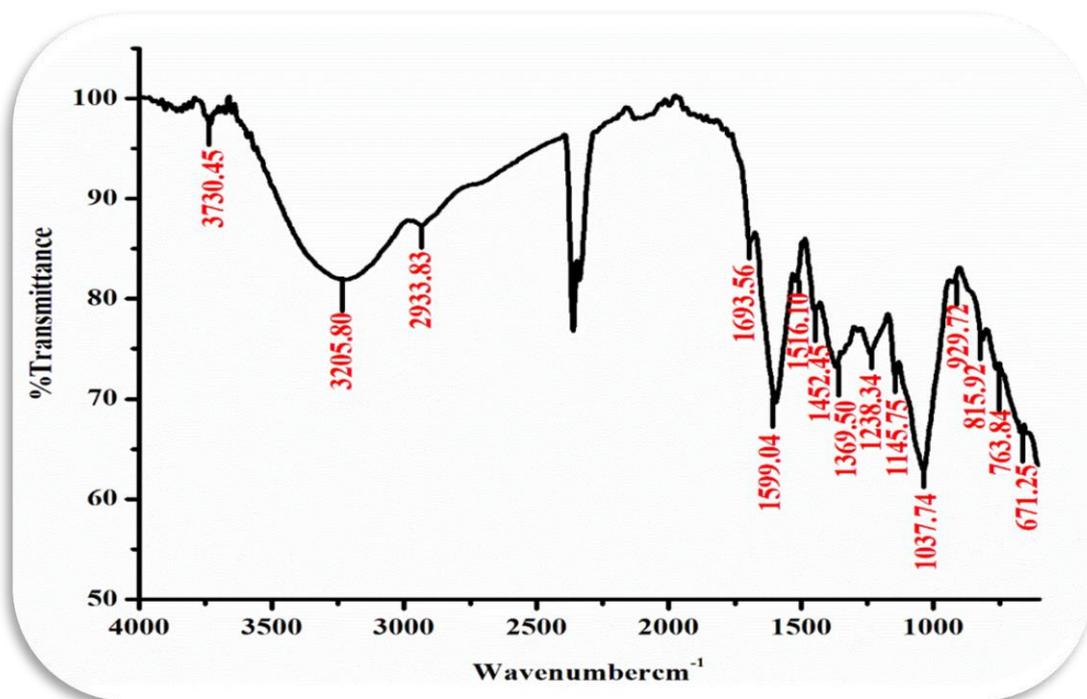


Fig. 37: FTIR spectra of green tea and *C. citratus* combination with a scan range from 600-4000cm⁻¹

6.4.3 Green tea and *C. flexuosus* combination (GT+CF)

The FTIR spectrum of GT+CF binary combination as given in **Fig. 38**, showed peaks at 3738.17 cm⁻¹ and 3848.12 cm⁻¹ exhibited (OH stretching). A strong and wide peak at 3265.59cm⁻¹ between the regions of 3000-3600 cm⁻¹ indicated the overlapping of hydroxyl group of alcohols or phenols, CH stretch of alkene, arenes and NH stretch of amine groups. The peaks at 1572.04 cm⁻¹ assigned (NH bending of amide and C=C aromatic groups), 1442.80 cm⁻¹ (C=C stretch of aromatic groups and CH bend of alkanes), 1371.43 cm⁻¹(C-H alkane bending), 1217.12 cm⁻¹ (CO stretch of carboxylic acids and CN stretch of amines), and 1051.24 cm⁻¹(CO stretching of phenol/alcohol/ester and ether) as given in **Table 17**.

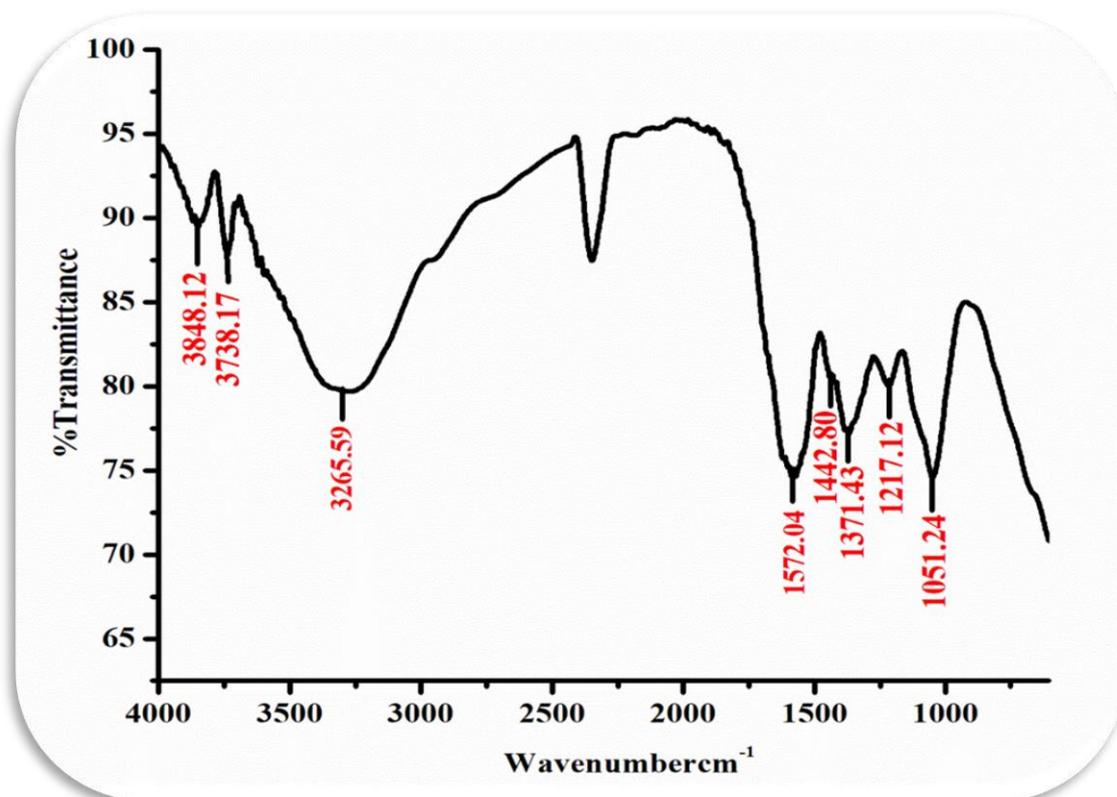


Fig. 38: FTIR spectra of green tea and *C. flexuosus* combination with a scan range from 600-4000cm⁻¹

6.4.4 Green tea and *H. rosa sinensis* combination (GT+HR)

The FTIR spectra of GT+HR binary combination depicted in **Fig. 39**, showed a peak at 3736.24 cm⁻¹ attributed free OH stretching and a broad band at 3213.51 cm⁻¹ in the region between 3000-3600 cm⁻¹ indicated the overlapping of hydroxyl group of alcohols or phenols, the CH stretch of alkene, arenes and NH stretch of amine groups. The absorption bands at 2933.83 attributed the CH/ OH stretching of alkanes and carboxylic acids, 1689.70 cm⁻¹ attributes the C=O stretching of carboxylic acids, 1595.18 cm⁻¹ exhibited the NH bending of amide and C=C stretching of aromatic groups, 1446.66 cm⁻¹ (C=C stretching of aromatic groups and C-H bending of alkanes), 1369.50 cm⁻¹ (CH bending of alkanes and C=C stretching of aromatic compounds), 1238.34 cm⁻¹ (CO stretching of carboxylic acids and CN stretching of amines), 1141.90 cm⁻¹ (CN stretching of amine), 1039.67 cm⁻¹ (CO stretching of phenol/alcohol/ester and ether), 815.92 cm⁻¹ (= CH bending of alkenes and C-H bending of aromatic compounds) and 651.96 cm⁻¹ (C-Cl stretching of alkyl halides as depicted in **Table 17**.

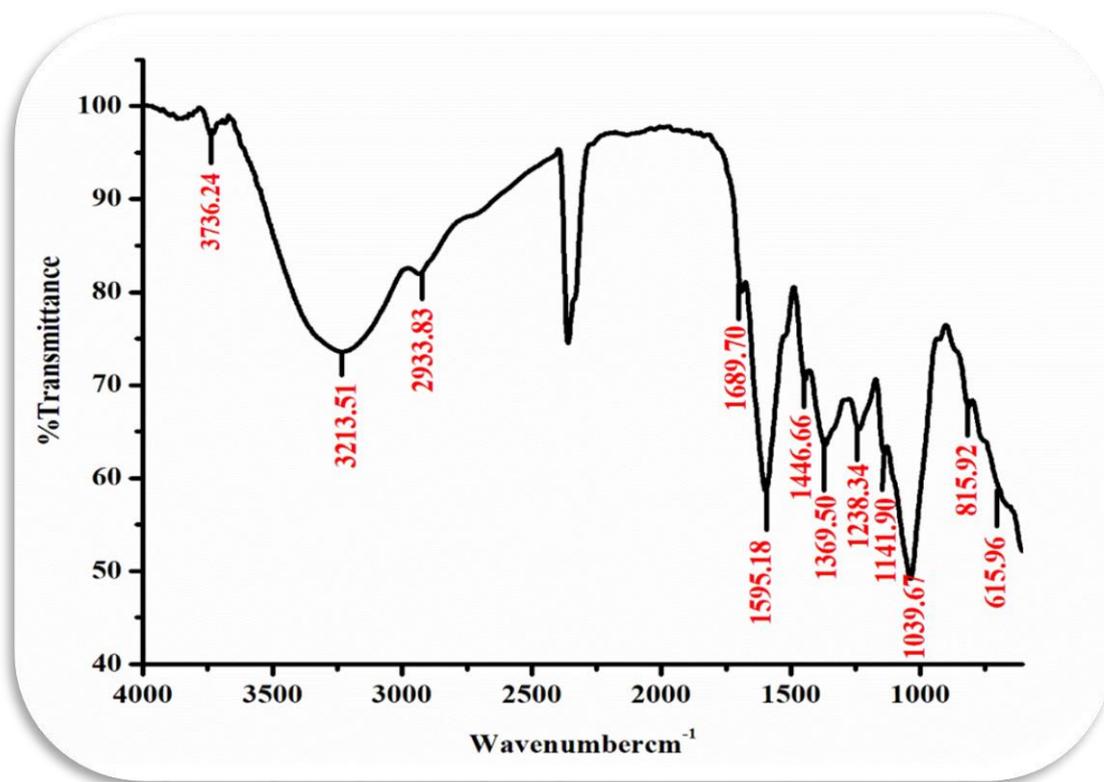


Fig. 39: FTIR spectra of green tea and *H. rosa sinensis* combination with a scan range from 600-4000cm⁻¹

Table 17: The absorption spectrum of aqueous extracts of green tea combinations, GT+OG, GT+CC, GT+CF and GT+HR.

| Functional groups | Absorption spectrum, frequency cm^{-1} | | | | |
|-------------------|---|------------------|---------------------------|------------------|---------------------------|
| | Component (Peaks) | GT + OG | GT + CC | GT + CF | GT + HR |
| | Wave number (cm^{-1}) | | | | |
| Alcohol/Phenol | O-H (Stretch , H-bounded) (3200-3600) | 3257.88 | 3205.80 | 3265.59 | 3213.51 |
| | O-H (Stretch, free) (3500-3800) | 3736.24, 3869.33 | 3730.45 | 3738.17, 3848.12 | 3736.24 |
| | C-O (Stretch) of alcohol (970-1250) | 1045.45 | 1037.74 | 1051.24 | 1039.67 |
| Carboxylic acids | C=O (Stretch) (1680-1710) | - | 1693.56 | - | 1689.70 |
| | O-H (Stretch) (2500-3500) | 2933.83 | 2933.83 | - | 2933.83 |
| | C-O (Stretch) (1210-1320) | 1217.12 | 1238.34 | 1217.12 | 1238.34 |
| Alkane | C-H (Stretch) (2850-3000) | 2933.83 | 2933.83 | - | 2933.83 |
| | C-H (Bending) (1350-1480) | 1352.14, 1442.80 | 1369.50, 1452.45 | 1371.43, 1442.80 | 1369.50, 1446.66 |
| Amine | N-H (Stretch) (2280-3380) | 3257.88 | 3205.80 | 3265.59 | 3213.51 |
| | C-N (stretch) (1080-1360) | 1217.12, 1352.14 | 1145.75, 1238.34 | 1217.12 | 1141.90, 1238.34 |
| Amide | N-H (Bending) (1550-1640) | 1602.90 | 1599.04 | 1572.04 | 1595.18 |
| Alkenes | =C-H (Bending) (675-1000) | - | 763.84, 815.92, 929.72 | - | 815.92 |
| Ether | C-O (Stretch) (1000-1300) | 1045.45 | 1037.74 | 1051.24 | 1039.67 |
| Ester | C-O (Stretch) (1000-1300) | 1045.45 | 1037.74 | 1051.24 | 1039.67 |
| Aromatic | C=C (Stretch) (1400-1600) | 1442.80 | 1452.45, 1599.04, 1516.10 | 1442.80, 1572.04 | 1369.50, 1446.66, 1595.18 |
| | C-H (Bend) (680-900) | - | 763.84, 815.92 | - | 815.92 |
| Alkyl halide | C-Cl (Stretch) (600-800) | - | 671.25, 763.84 | - | 651.96 |

GT, OG, CF, CC and HR represent Green tea, *O. gratissimum*, *C. flexuosus*, *C. citratus* and *H. rosa-sinensis*.

6.4.5 Comparison of FTIR spectra of GT combinations

The GT combinations demonstrated similar spectra, as that of GT single infusion except few additional peaks in the region 3600–4000 cm^{-1} . It was observed that GT+OG demonstrated the most intense peak in region 3000-3600 cm^{-1} followed by GT+HR, GT+CF and GT+CC. In the region 1500-2000 cm^{-1} GT+OG and GT+HR demonstrated similar peak intensity followed by GT+CC and GT+CF. However, in the region 800-1200 cm^{-1} , GT+OG and GT+HT displayed the similar peak intensity followed by GT+CC and GT+CF. When this peak was compared to IR spectrum of GT, OG and CF alone, these stretching become narrow with a decrease in the peak intensity and broadening as given in **Fig. 40**.

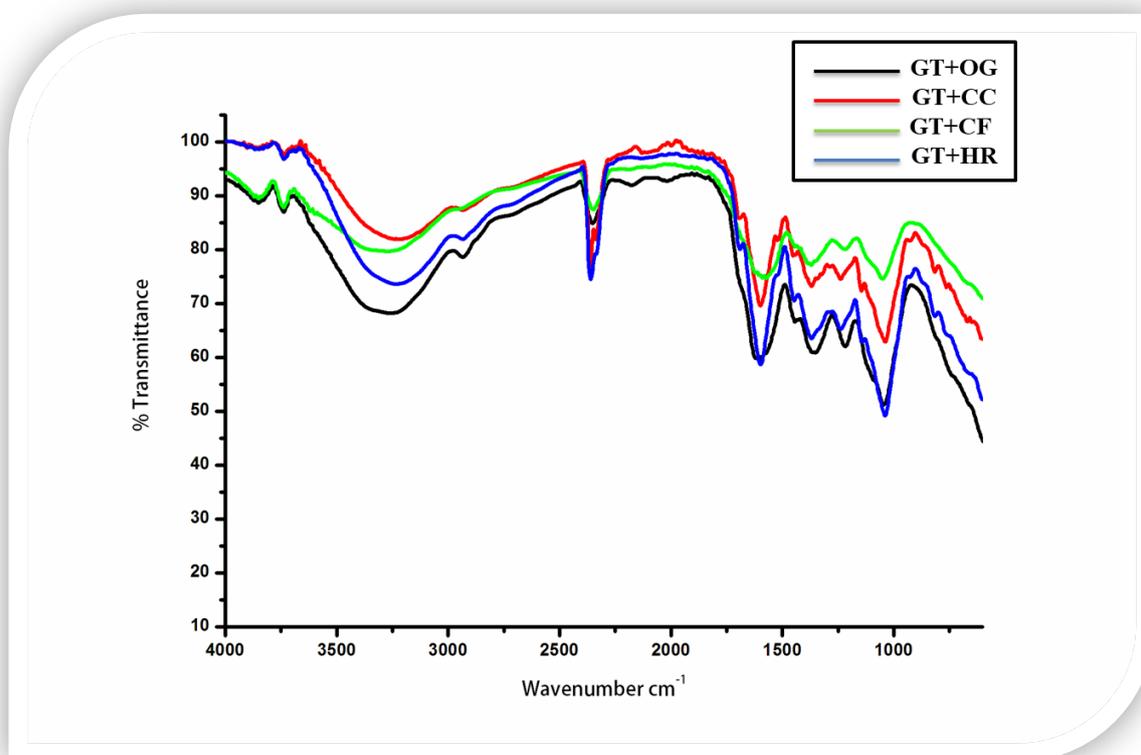


Fig. 40: Comparison of FTIR spectra of all GT combinations with a scan range from 600-4000 cm^{-1}

6.4.6 Correlation between FTIR peak intensity and total phenolic content/antioxidant activity of green tea binary combinations

A very high positive correlation to low positive correlation was observed between absorbance and antioxidant activities [high positive correlation for haemolysis ($r = 0.996$), DPPH ($r = 0.907$) and NO ($r = 0.747$), moderate positive correlation for LPO ($r = 0.530$), low positive

correlation for ABTS ($r = 0.434$)] whereas negligible correlation ($r = -0.189$) between TPC and absorbance of the peak in the selected region as shown in **Table 18**.

Table 18: A correlation analysis between intensity of absorbance peak ($3206-3267\text{cm}^{-1}$) and total phenolic content or antioxidant activity of binary combinations.

| Parameter | Correlation coefficient (r) | Type of correlation |
|------------|-----------------------------|---------------------|
| TPC | -0.189 | Negligible |
| DPPH | 0.907 | High (+ve) |
| ABTS | 0.434 | Low (+ve) |
| NO | 0.747 | High (+ve) |
| LPO | 0.530 | Moderate (+ve) |
| Haemolysis | 0.996 | Very high (+ve) |

Whereas, DPPH; 2, 2-diphenyl-1-picrylhydrazyl, ABTS; [(2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)], NO; nitric oxide, LPO; lipid peroxidation, TPC; Total phenolic content, +ve (positive) and FTIR (Fourier transform infrared spectrum).

6.4.7 UV- VIS absorption spectra of individual samples

The UV-VIS absorbance spectra of aqueous infusions of green tea (GT), *O.gratissimum* (OG), *C.citratus* (CC), *C. flexuosus* (CF) and *H. rosa sinensis* (HR) were recorded in the range 200–800 nm as shown in **Fig. 41**. Among all the extracts analyzed, only green tea demonstrated an intense peak in the region between 250-300 nm and other infusions showed a very low peak in this region. This peak in GT may be due to catechin content or presence of caffeine that are absent in herbal teas. GT sample displayed maximum absorbance ($A=1.130$) at wavelength ($\lambda_{\text{max}} = 271$ nm). The graph clearly depicts that the intensity of all samples declined at wavelength greater than 400nm.

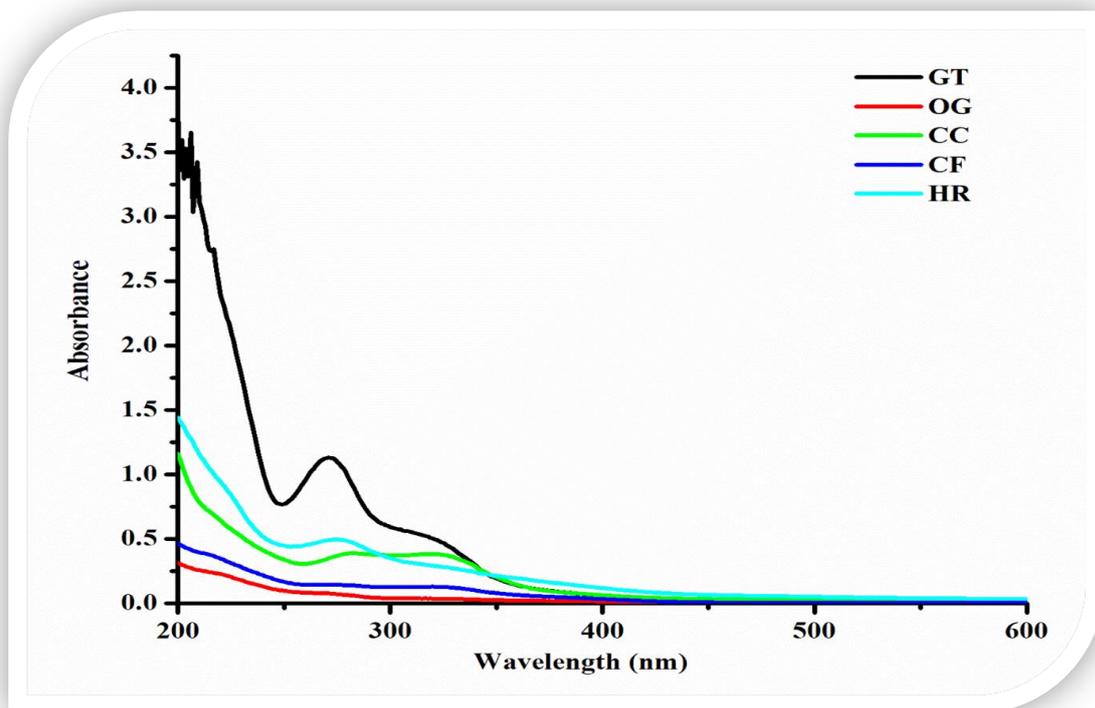


Fig. 41. UV–vis spectra of green tea (GT), *O. gratissimum* (OG), *C. citratus* (CC), *C. flexuosus* (CF) and *H. rosa-sinensis* (HR) extracts.

6.4.7.1 Correlation analysis between ultraviolet region absorption peak and antioxidant activity of individual samples

The correlation analysis between ultraviolet region absorbance peak (272nm) and total phenolic content or antioxidant activities of individual infusions exhibited a high positive correlation ($r = 0.867$) between TPC and absorbance of different samples by UV-VIS analysis whereas negligible to very high negative correlation was found between the EC_{50} and absorbance of all infusions in various assays performed such as LPO ($r = -0.279$), DPPH ($r = -0.749$), ABTS ($r = -0.757$), NO ($r = -0.822$) and Haemolysis ($r = -0.926$) as depicted in **Table 19**.

Table 19: A correlation analysis between ultraviolet region absorbance peak (272nm) and total phenolic content or antioxidant activities of individual infusions.

| Parameter | Correlation coefficient (r) | Type of correlation |
|------------|-----------------------------|---------------------|
| TPC | 0.867 | High (+ve) |
| DPPH | -0.749 | High (-ve) |
| ABTS | -0.757 | High (-ve) |
| NO | -0.822 | High (-ve) |
| LPO | -0.289 | Negligible |
| Haemolysis | -0.926 | Very high (-ve) |

Whereas DPPH; 2, 2-diphenyl-1-picrylhydrazyl, ABTS; [(2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)], NO; nitric oxide, LPO; lipid peroxidation, TPC; Total phenolic content, +ve (positive), -ve (negative) and TPC; Total phenolic content.

6.4.8 Ultraviolet-visible (UV-VIS) spectra of GT combinations

The Ultraviolet-visible spectra of all the GT combinations (GT+OG, GT+CC, GT+CF and GT+HR) are shown in **Fig. 42**. All GT combinations demonstrated an intense peak near the region of 250-300 nm, and GT+OG demonstrated the highest absorption peak followed by GT+HR, GT+CF and GT+CC. The absorbance of the GT+ OG was highest ($A = 1.121$) at the wavelength ($\lambda_{\max} = 272$ nm), whereas GT+HR ($A = 1.056$), GT+CF ($A = 1.052$) and GT+CC ($A = 0.902$) at the wavelength ($\lambda_{\max} = 271$ nm), respectively.

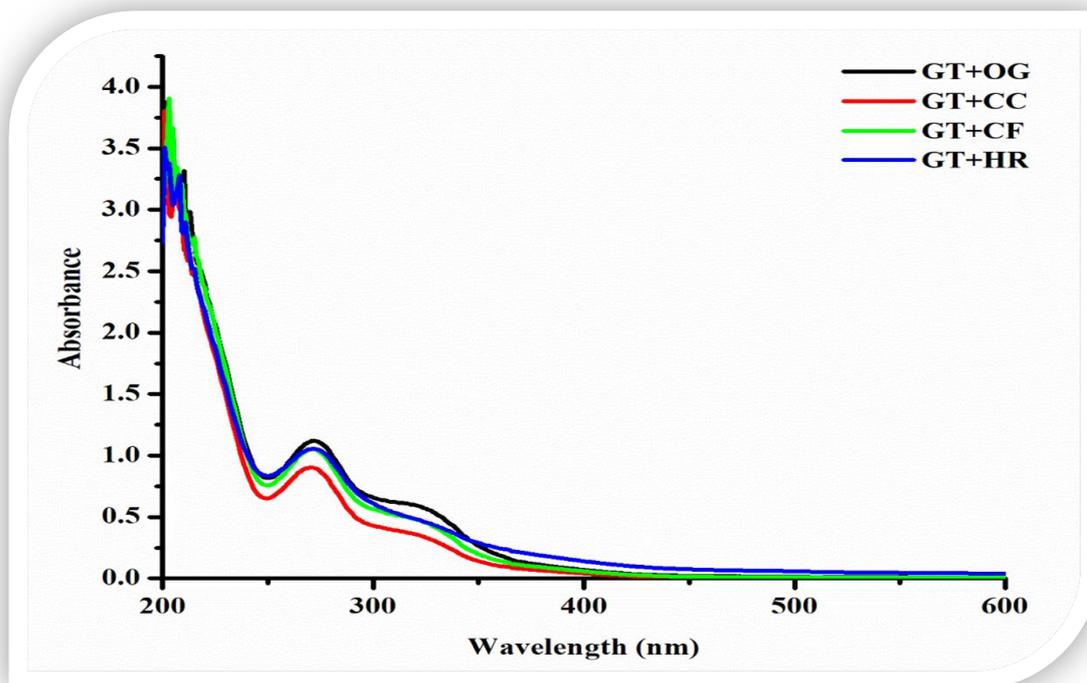


Fig. 42. UV–VIS spectra of green tea combinations. GT, OG, CF, CC and HR represent Green tea, *O. gratissimum*, *C. flexuosus*, *C. citratus* and *H. rosa-sinensis*.

6.4.8.1 Correlation analysis between ultraviolet region absorption peak and antioxidant activities of green tea combinations

A moderate positive correlation ($r = 0.688$) observed between TPC and absorbance of different GT combinations, but negligible to high negative correlation between (antioxidant activity (EC_{50})) and absorbance of different samples in various assays like ABTS ($r = 0.002$), LPO ($r = - 0.153$), NO ($r = - 0.352$), DPPH ($r = - 0.553$) and Haemolysis ($r = - 0.799$) as depicted in **Table 20**.

Table 20: A correlation analysis between ultraviolet region absorbance peak (272nm) and the total phenolic content or antioxidant activities of green tea combinations.

| Parameter | Correlation coefficient (r) | Type of correlation |
|------------|-----------------------------|---------------------|
| TPC | 0.668 | Moderate (+ve) |
| DPPH | -0.553 | Moderate (+ve) |
| ABTS | 0.002 | Negligible |
| NO | -0.352 | Low (-ve) |
| LPO | -0.153 | Negligible |
| Haemolysis | -0.799 | High (-ve) |

Whereas DPPH; 2, 2-diphenyl-1-picrylhydrazyl, ABTS; [(2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)], NO; nitric oxide, LPO; lipid peroxidation, +ve (positive), -ve (negative) and TPC; Total phenolic content.

6.5 Antioxidant activity of green tea (GT) and *O. gratissimum* (OG) combination at different ratios

GT and OG combination illustrated highest antioxidant capacity among the evaluated green tea combinations. The GT and OG combination were further scrutinized at different ratios by determining antioxidant activities using cell free tests such as DPPH, ABTS, NO and *ex vivo* assays like LPO and haemolysis by calculating EC₅₀. The less the EC₅₀ value, the more is the antioxidant ability. The TPC/TFC of GT and OG aqueous infusions alone, and as binary mixture at different ratios was also estimated.

6.5.1 DPPH antioxidant test

The DPPH quenching potential of different infusions was ranked upon EC₅₀ values and the decreasing order of EC₅₀ are as follows: *O. gratissimum* (OG), 1:2, 1:3, 3:1, 2:1, 1:1 and GT, respectively. GT alone and its binary combinations (3:1, 2:1 and 1:1) exhibited similar ($p \geq 0.05$) but higher DPPH scavenging ability as compared to other combinations (1:2 and 1:3) and OG ($p \leq 0.05$) as displayed in **Fig. 43** and **Table 21**.

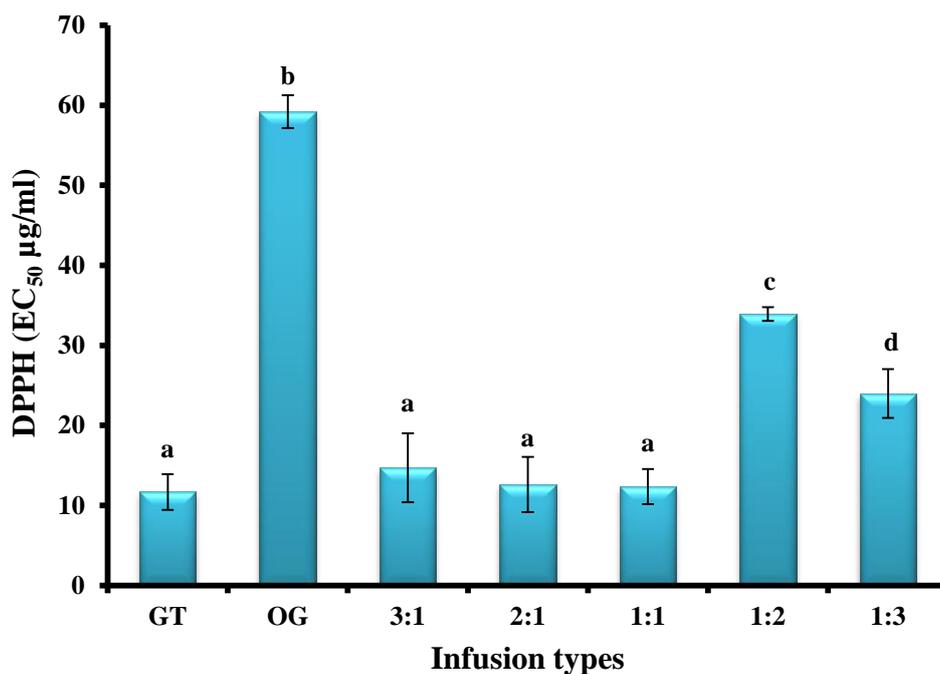


Fig. 43: DPPH radical scavenging capacity of GT, OG and their ratios (3:1, 2:1, 1:1, 1:2 and 1:3). The data is shown as MEAN ± S.D of three 3 independent tests (each with triplicates for each test point). The dissimilar alphabets revealed significant difference ($p \leq 0.05$) among the EC₅₀ of different infusions. EC₅₀(Effective concentration causing 50% scavenging activity).

6.5.2 ABTS antioxidant test

The antioxidant potential of various infusions with different proportion of green tea and *O. gratissimum* against ABTS radical is depicted in **Fig. 44**. GT alone and its binary combinations (1:1, 2:1 and 3:1) demonstrated similar ($p \geq 0.05$), but higher ABTS scavenging potential compared to other combinations (1:2 and 1:3) and OG ($p \leq 0.05$) **Table 21**.

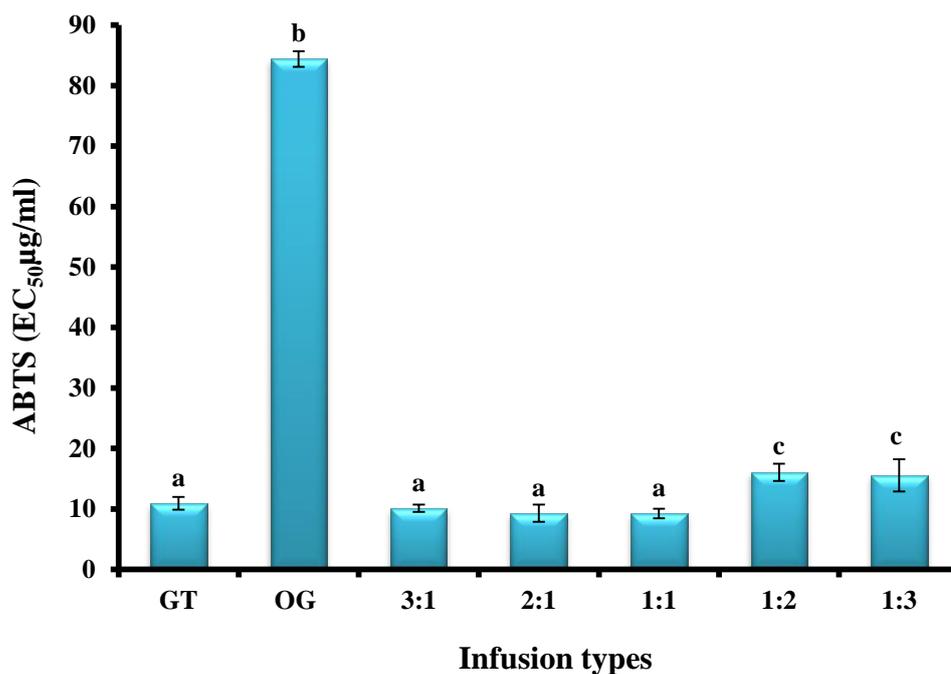


Fig. 44: ABTS radical scavenging capacity of GT, OG and their ratios (3:1, 2:1, 1:1, 1:2 and 1:3). The data is shown as MEAN \pm S.D of three 3 independent tests (each with triplicates for each test point). The dissimilar alphabets revealed significant difference ($p \leq 0.05$) among the EC₅₀ of different infusions. EC₅₀ (Effective concentration causing 50% scavenging activity).

6.5.3 Nitric oxide (NO) test

In the assay of nitric oxide the binary combination (1:1) demonstrated greater ($p \leq 0.05$) NO radical scavenging capacity as compared to other infusions (2:1, GT, 1:3, 1:2, 3:1, and OG). OG displayed lowest ($p \leq 0.05$) radical scavenging potential with highest EC₅₀ value as illustrated in **Fig. 45** and **Table 21**.

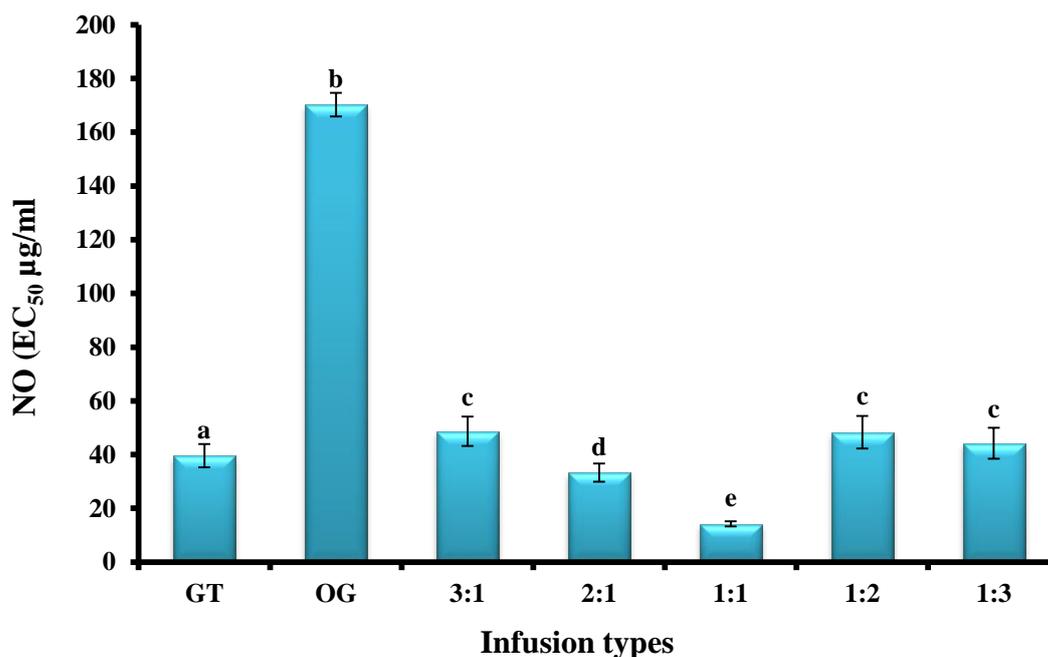


Fig. 45: Nitrite radical scavenging capacity of GT, OG and their ratios (3:1, 2:1, 1:1, 1:2 and 1:3). The data is shown as MEAN \pm S.D of three independent tests (each with triplicates for each test point). The dissimilar alphabets revealed significant difference ($p \leq 0.05$) among the EC₅₀ of different infusions. EC₅₀ (Effective concentration causing 50% scavenging activity).

6.5.4 Anti-lipid peroxidation assay

The inhibitory activity of various aqueous infusions against FeSO₄-induced lipid peroxidation in chick liver was evaluated. The binary combination (3:1, 2:1 and 1:1) revealed similar ($p \geq 0.05$) but higher anti-lipid peroxidation ability as compared to other infusions 1:2, 1:3, GT and OG ($p \leq 0.05$) as depicted in **Fig. 46** and **Table 21**.

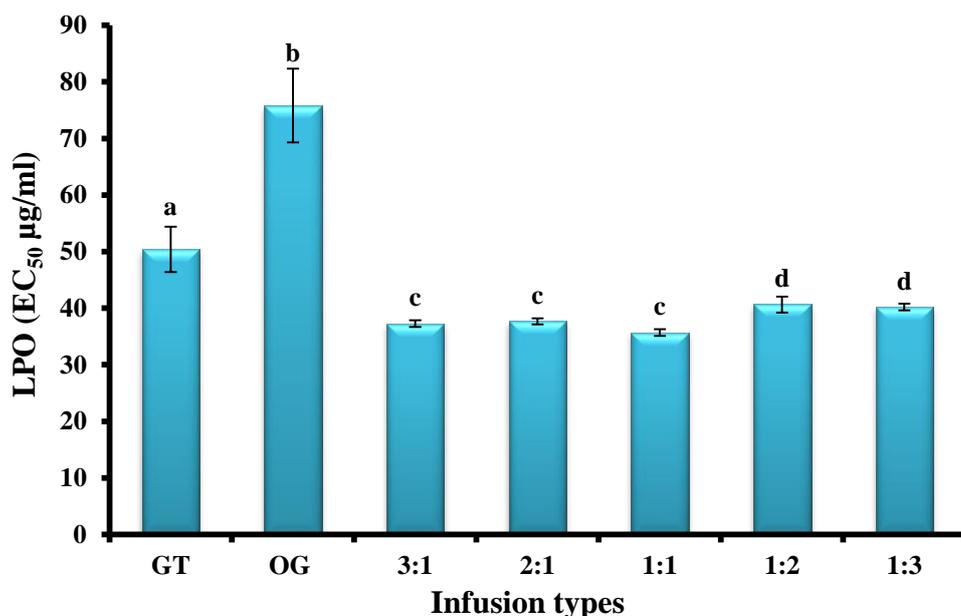


Fig. 46: Anti-lipid peroxidation capacity of GT, OG and their ratios (3:1, 2:1, 1:1, 1:2 and 1:3). The data is shown as MEAN \pm S.D of three 3 independent tests (each with triplicates for each test point). The dissimilar alphabets revealed significant difference ($p \leq 0.05$) among the EC₅₀ of different infusions. EC₅₀ (Effective concentration causing 50% scavenging activity).

6.5.5 Anti-haemolysis assay

The inhibitory activity of various aqueous infusions on hydrogen peroxide induced erythrocyte haemolysis was evaluated as displayed in **Fig. 47**. It was found that combination (1:1) demonstrated higher ($p \leq 0.05$) anti-haemolytic activity with lowest EC₅₀ as compared to other infusions whereas OG showed the lowest anti-haemolytic activity among all infusions **Table 21**.

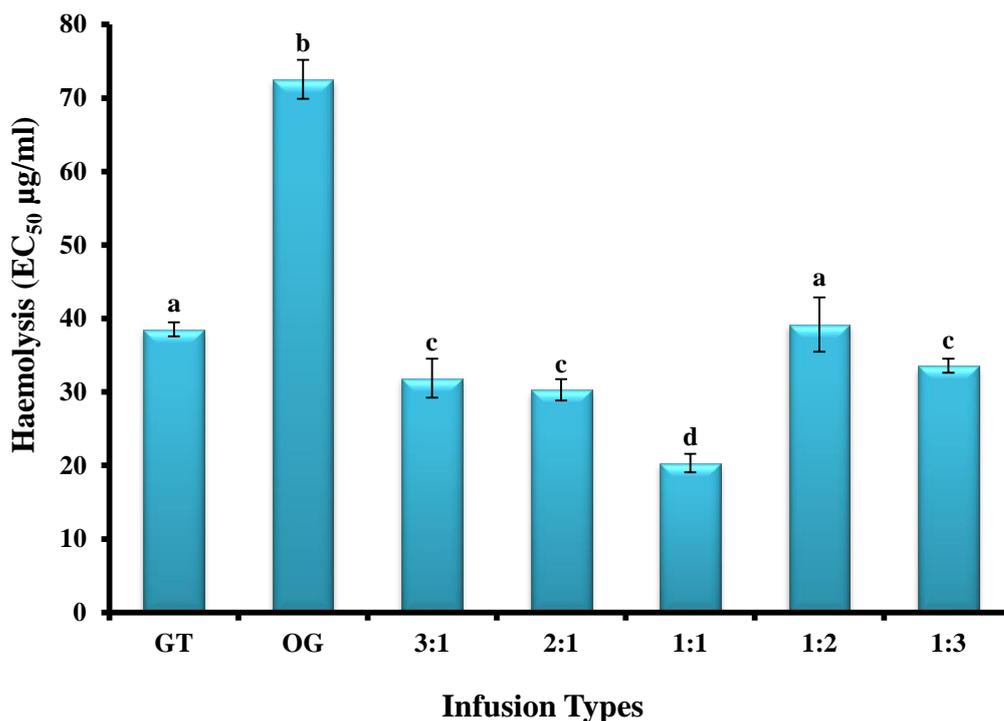


Fig. 47: Anti-Haemolytic potential of GT, OG and their ratios (3:1, 2:1, 1:1, 1:2 and 1:3). The data is shown as MEAN \pm S.D of three 3 independent tests (each with triplicates for each test point). The dissimilar alphabets revealed significant difference ($p \leq 0.05$) among the EC₅₀ of different infusions. EC₅₀ (Effective concentration causing 50% scavenging activity).

6.5.6 Interactions by combination index and isobolographic method

The antioxidant interaction of GT and OG combination (GT+OG) at different ratios (3:1, 2:1, 1:1, 1:2, and 1:3) was determined applying combination index and isobolograms.

6.5.6.1 Interactions of GT+OG combinations at various ratios in different assays

In DPPH assay, GT and OG combination (1:1) exhibited lowest CI value ($p \leq 0.05$) and highest synergism as compared to 2:1 \approx 1:3, whereas 3:1 displayed additive, and 1:2 antagonistic interactions as illustrated in **Table 21**. The isobolographic analysis showed that for GT+OG combinations (2:1, 1:1 and 1:3), the EC₅₀ value lies below the line of additivity indicates synergism (**Fig. 48b, c and e**), whereas it lies on the line of additivity for 3:1 that means nearly additive effect (**Fig 48a**) and above the line of additivity for 1:2 which showed the antagonistic interaction (**Fig. 48d**)

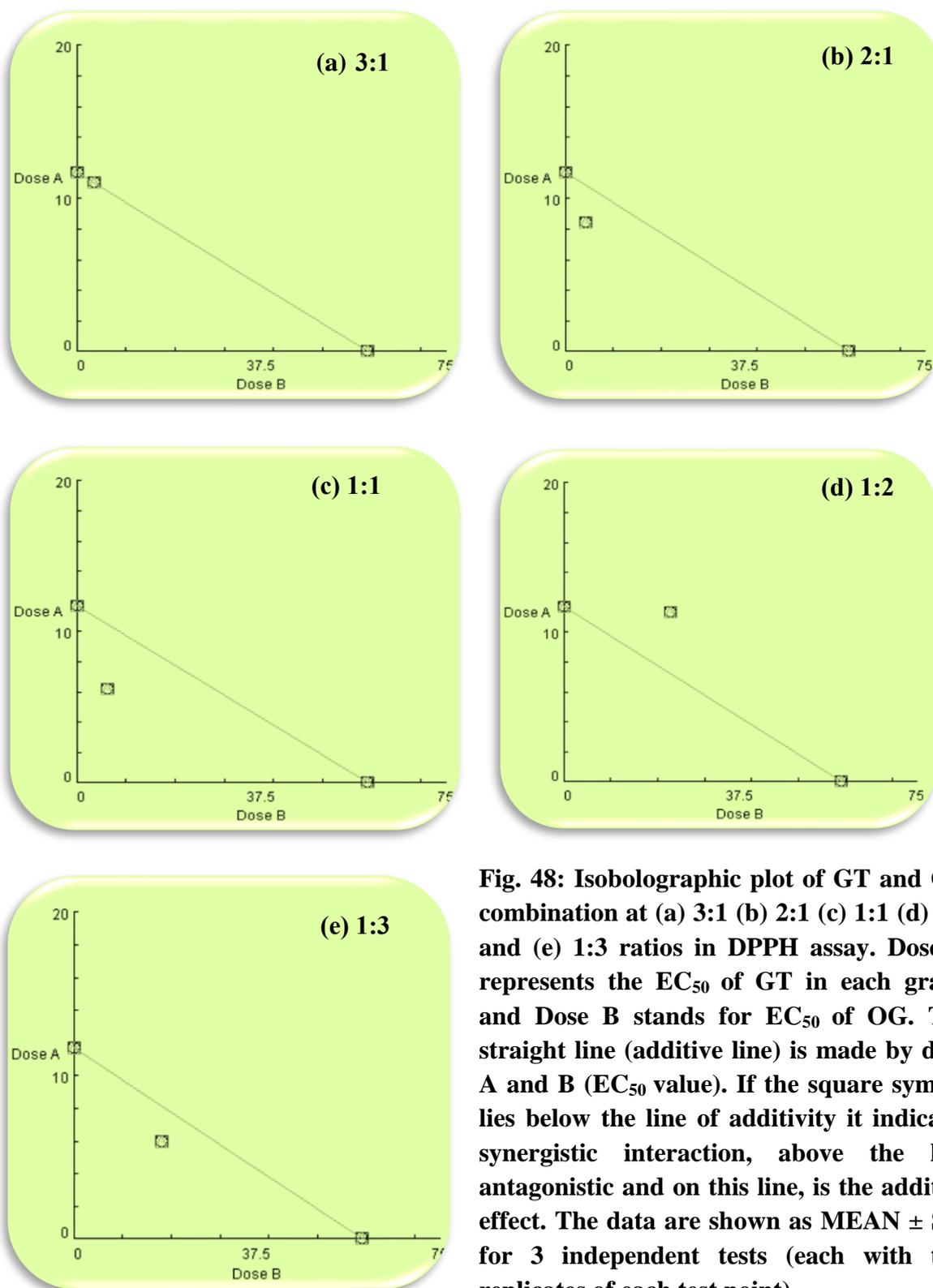
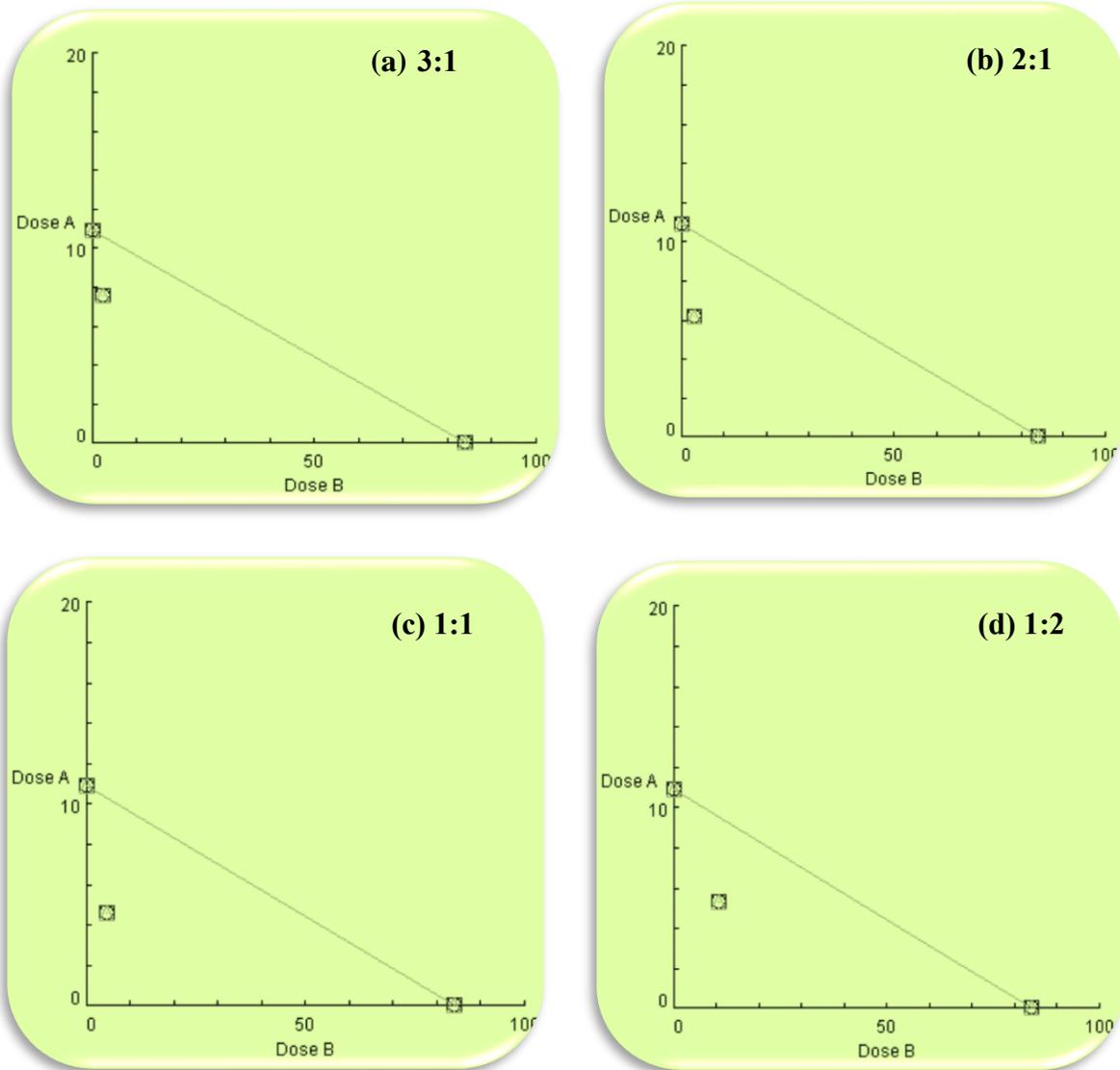


Fig. 48: Isobolographic plot of GT and OG combination at (a) 3:1 (b) 2:1 (c) 1:1 (d) 1:2 and (e) 1:3 ratios in DPPH assay. Dose A represents the EC₅₀ of GT in each graph and Dose B stands for EC₅₀ of OG. The straight line (additive line) is made by dose A and B (EC₅₀ value). If the square symbol lies below the line of additivity it indicates synergistic interaction, above the line antagonistic and on this line, is the additive effect. The data are shown as MEAN ± S.D for 3 independent tests (each with two replicates of each test point).

In ABTS radical scavenging assay, the CI values of GT+OG combination (1:1 and 1:3) displayed similar ($p \geq 0.05$) but lower ($p \leq 0.05$) CI value and maximum synergism as compared to other combinations (3:1, 2:1 and 1:2) as given in **Table 21**. In this assay, the

isobolograms revealed that EC_{50} value lies below the line of additivity in all combinations (3:1, 2:1, 1:1, 1:2 and 1:3) that means demonstrating synergistic interaction as depicted in **Fig.49a-e**.



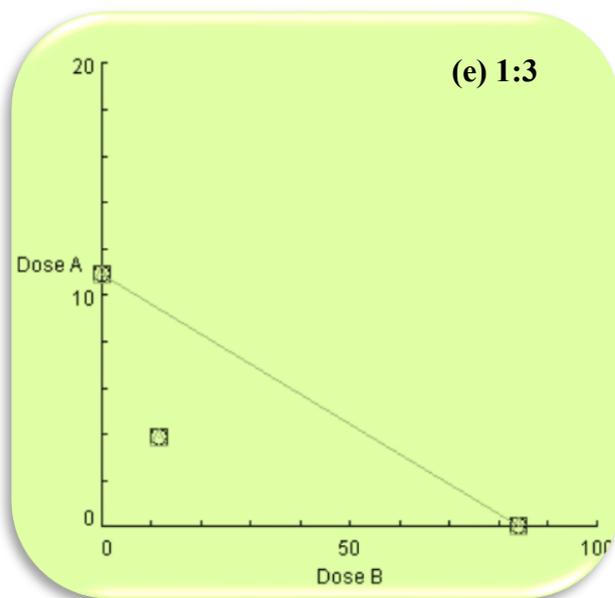
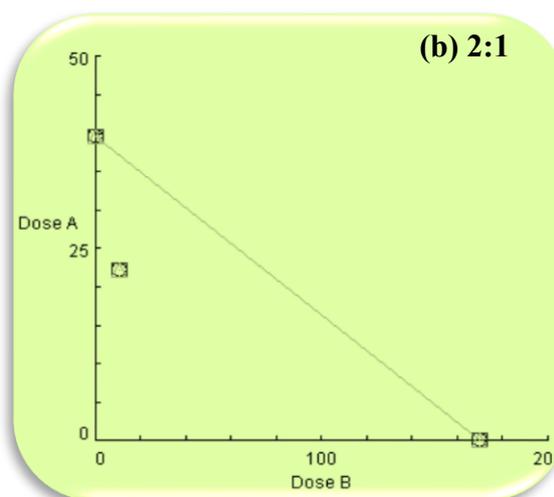
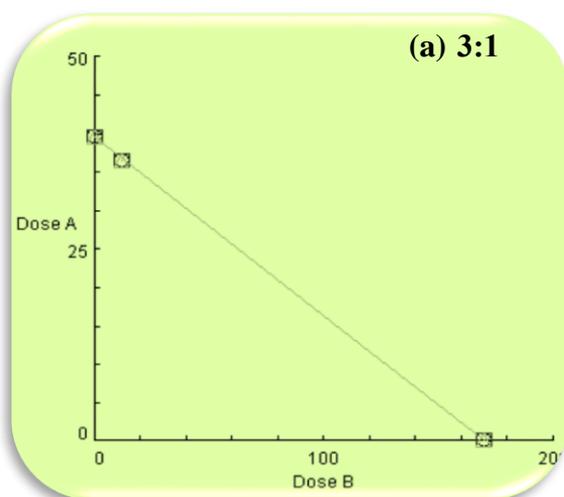


Fig. 49: Isobolographic plot of GTand OG combination at (a) 3:1 (b) 2:1 (c) 1:1 (d) 1:2 and (e) 1:3 ratios in ABTS assay. Dose A represents the EC₅₀ of GT in each graph and Dose B stands for EC₅₀ of OG. The straight line (additive line) is made by dose A and B (EC₅₀ value). If the square symbol lies below the line of additivity it indicates synergistic interaction, above the line antagonistic and on this line, is the additive effect. The data are shown as MEAN ± S.D for 3 independent tests (each with two replicates of each test point).

In nitrite radical scavenging test, the CI value of binary combinations was in the following increasing order; 1:1, 1:3, 1:2, 2:1 (synergistic interactions) and 3:1 (nearly additive interaction) as given in **Table 21**. It is observed that combination (1:1) displayed lowest CI value with strong synergism as compared to other proportions (1:3, 1:2 and 2:1). In a binary combination (3:1), the EC₅₀ value is on the line of additivity demonstrated the nearly additive interaction **Fig. 50a**, whereas combinations (2:1, 1:2, 1:3 and 1:1) the EC₅₀ value lies below the line of additivity demonstrating synergistic interaction as depicted in **Fig. 50b-e**.



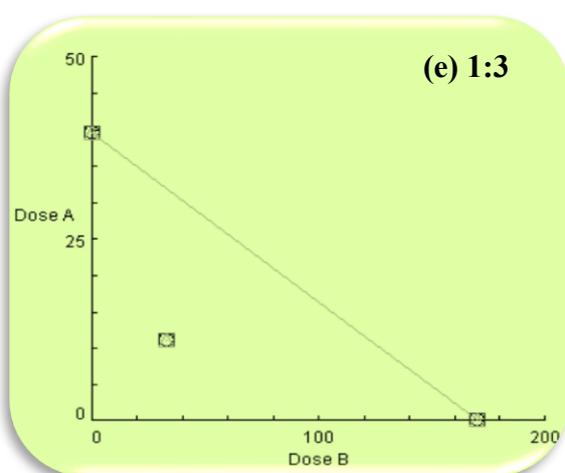
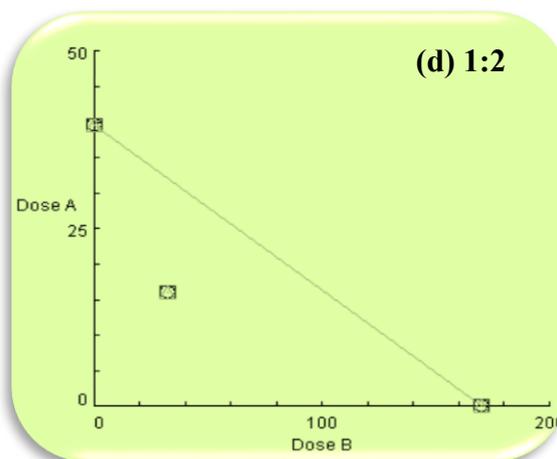
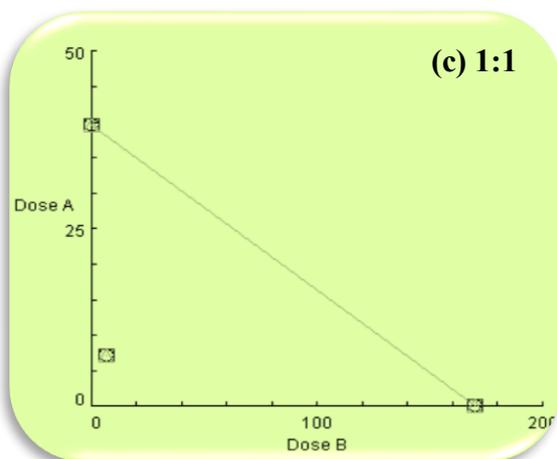


Fig. 50: Isobolographic plot of GTand OG combination at (a) 3:1 (b) 2:1 (c) 1:1 (d) 1:2 and (e) 1:3 ratios in NO assay. Dose A represents the EC₅₀ of GT in each graph and Dose B stands for EC₅₀ of OG. The straight line (additive line) is made by dose A and B (EC₅₀ value). If the square symbol lies below the line of additivity it indicates synergistic interaction, above the line antagonistic and on this line, is the additive effect. The data are shown as MEAN \pm S.D for 3 independent tests (each with two replicates of each test point).

In anti-lipid peroxidation assay, GT+OG combinations at all ratios displayed synergistic interactions with similar ($p \geq 0.05$) CI value as displayed in **Table 21**. In current assay the EC₅₀ values of all binary combinations (3:1, 2:1, 1:1, 1:2 and 1:3) lie below the line of additivity thus showing synergistic interaction as given in **Fig. 51a-e**.

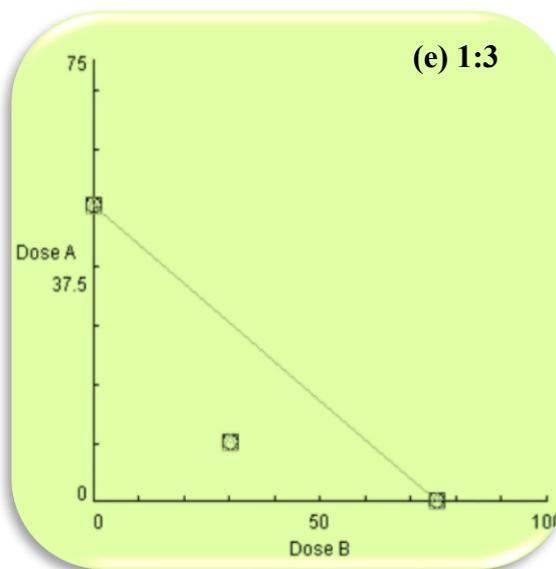
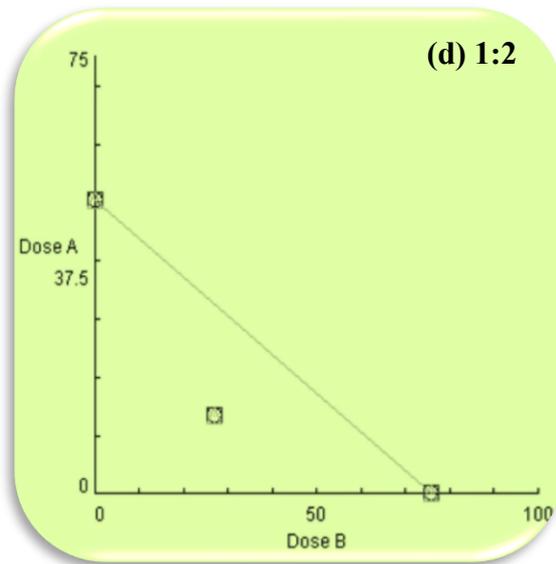
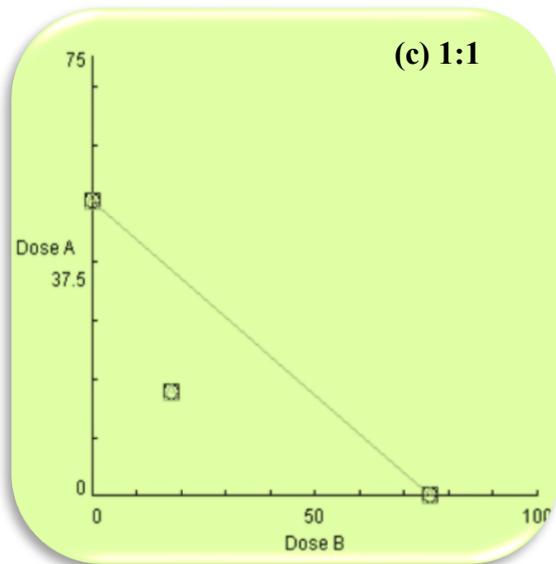
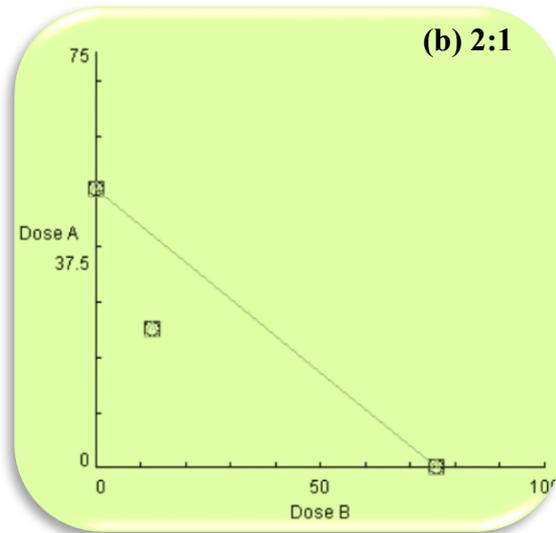
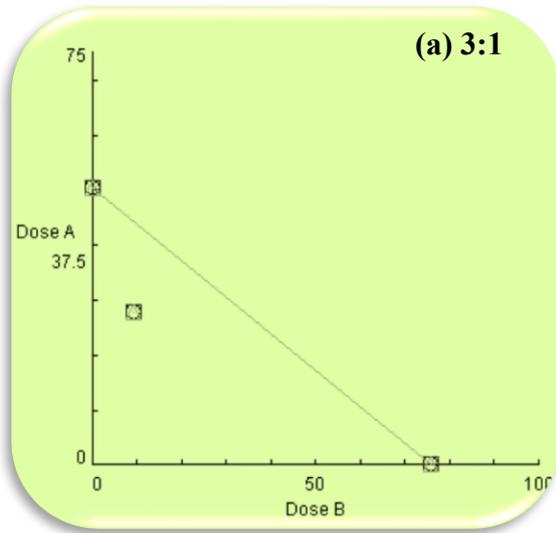
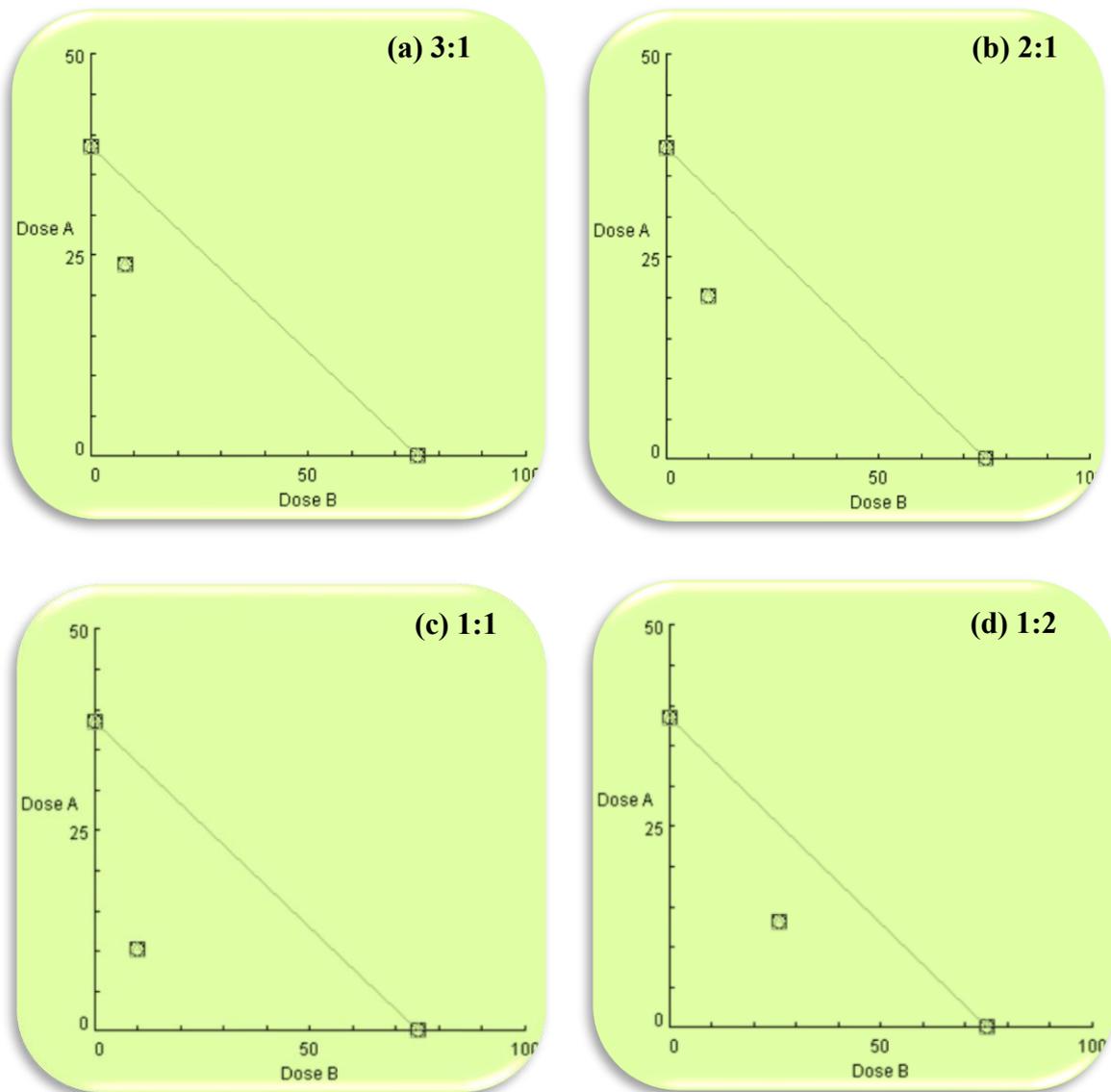


Fig. 51: Isobolographic plot of GT and OG combination at (a) 3:1 (b) 2:1 (c) 1:1 (d) 1:2 and (e) 1:3 ratios in LPO assay. Dose A represents the EC₅₀ of GT in each graph and Dose B stands for EC₅₀ of OG. The straight line (additive line) is made by dose A and B (EC₅₀ value). If the square symbol lies below the line of additivity it indicates synergistic interaction, above the line antagonistic and on this line, is the additive effect. The data are shown as MEAN ± S.D for 3 independent tests (each with two replicates of each test point).

In anti-haemolysis assay, interaction ranged from synergism to moderate synergism, the combination (1:1) revealed maximum synergism with lowest ($p \leq 0.05$) CI value than other samples as shown in **Table 21**. The isobolographic study demonstrated that EC_{50} value of all binary mixtures lie below the line of additivity thus demonstrating synergistic interactions as depicted in **Fig. 52a-e**.



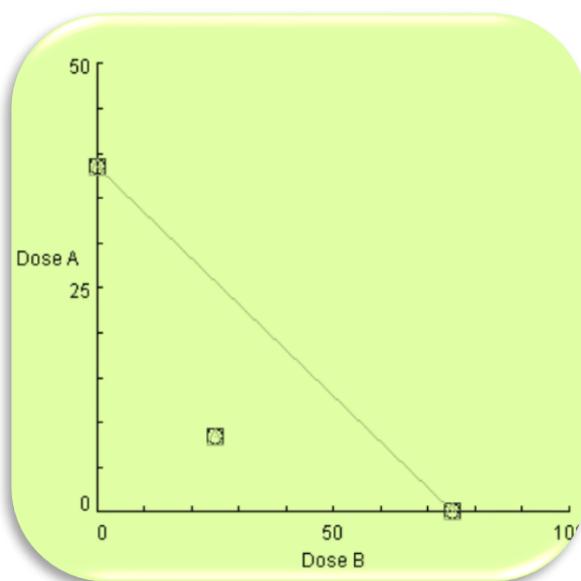


Fig. 52: Isobolographic plot of GT and OG combination at (a) 3:1(b) 2:1 (c) 1:1 (d) 1:2 and (e) 1:3 ratios in anti- haemolytic assay. Dose A represents the EC₅₀ of GT in each graph and Dose B stands for EC₅₀ of OG. The straight line (additive line) is made by dose A and B (EC₅₀ value). If the square symbol lies below the line of additivity it indicates synergistic interaction, above the line antagonistic and on this line, is the additive effect. The data are shown as MEAN ± S.D for 3 independent tests (each with two replicates of each test point).

Table 21: EC₅₀, combination index (CI), synergistic, additive and antagonistic antioxidant interactions of GT and OG combination at different ratios

| Assay types | Ratio (GT: OG) | EC ₅₀ (µg/ml) | CI at EC ₅₀ | Description |
|-------------|-------------------|---------------------------|------------------------|---------------------|
| DPPH | 1:0 | 11.68 ± 2.24 ^a | - | - |
| | 3:1 | 14.70 ± 4.30 ^a | 1.00 ^c | Nearly additive |
| | 2:1 | 12.61 ± 3.45 ^a | 0.79 ^b | Moderate synergism |
| | 1:1 | 12.36 ± 2.19 ^a | 0.63 ^a | Synergism |
| | 1:2 | 33.93 ± 0.85 ^c | 1.35 ^c | Moderate antagonism |
| | 1:3 | 23.97 ± 3.08 ^d | 0.81 ^b | Moderate synergism |
| | 0:1 | 59.21 ± 2.06 ^b | - | - |
| ABTS | 1:0 | 10.88 ± 1.07 ^a | - | - |
| | 3:1 | 10.10 ± 0.59 ^a | 0.72 ^c | Moderate synergism |
| | 2:1 | 9.27 ± 1.43 ^a | 0.60 ^b | Synergism |
| | 1:1 | 9.24 ± 0.78 ^a | 0.48 ^a | Synergism |
| | 1:2 | 16.06 ± 1.43 ^c | 0.62 ^b | Synergism |
| | 1:3 | 15.56 ± 2.68 ^c | 0.49 ^a | Synergism |
| | 0:1 | 84.07 ± 1.31 ^b | - | - |
| | 1:0 | 39.59 ± 4.37 ^a | - | - |
| | 3:1 | 48.70 ± 5.41 ^c | 0.99 ^d | Nearly additive |

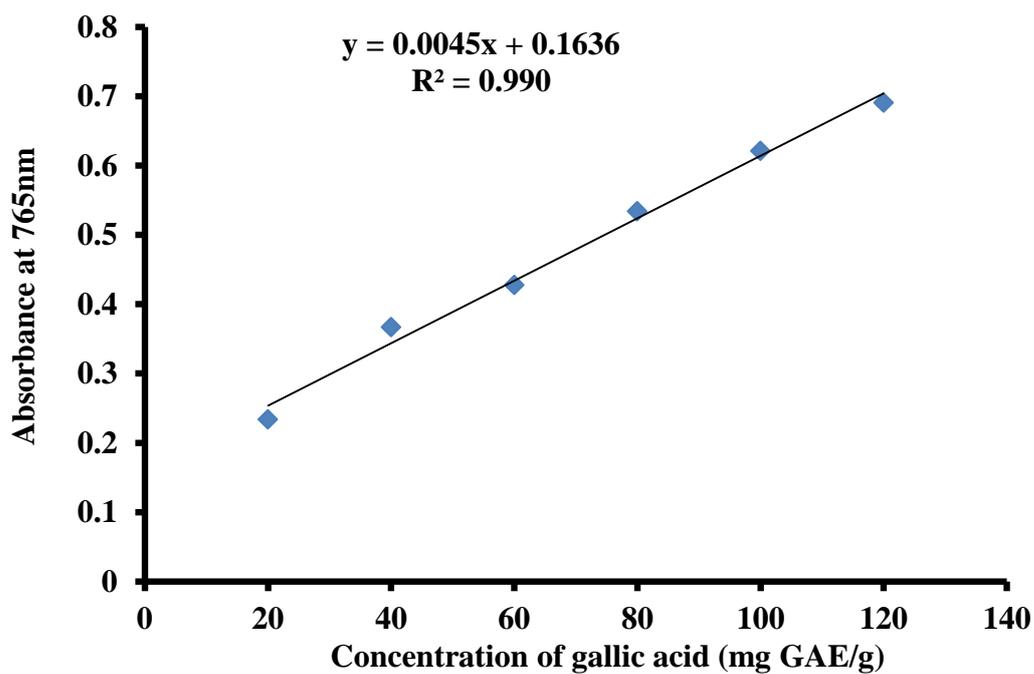
| | | | | |
|-------------------|-----|----------------------------|-------------------|--------------------|
| NO | 2:1 | 33.31 ± 3.42 ^d | 0.62 ^c | Synergism |
| | 1:1 | 14.21 ± 0.97 ^e | 0.22 ^a | Strong synergism |
| | 1:2 | 48.33 ± 6.04 ^c | 0.59 ^c | Synergism |
| | 1:3 | 44.21 ± 5.76 ^c | 0.47 ^b | Synergism |
| | 0:1 | 170.25 ± 4.39 ^b | - | - |
| | 1:0 | 50.40 ± 4.03 ^a | - | - |
| | 3:1 | 37.26 ± 0.60 ^c | 0.67 ^a | Synergism |
| LPO | 2:1 | 37.67 ± 0.55 ^c | 0.66 ^a | Synergism |
| | 1:1 | 35.69 ± 0.60 ^c | 0.58 ^a | Synergism |
| | 1:2 | 40.65 ± 1.41 ^d | 0.62 ^a | Synergism |
| | 1:3 | 40.22 ± 0.59 ^d | 0.59 ^a | Synergism |
| | 0:1 | 75.81 ± 6.50 ^b | - | - |
| | 1:0 | 38.52 ± 0.95 ^a | - | - |
| | 3:1 | 31.87 ± 2.66 ^c | 0.72 ^d | Moderate synergism |
| Haemolysis | 2:1 | 30.29 ± 1.45 ^c | 0.65 ^c | Synergism |
| | 1:1 | 20.32 ± 1.26 ^d | 0.39 ^a | Synergism |
| | 1:2 | 39.17 ± 3.69 ^a | 0.68 ^c | Synergism |
| | 1:3 | 33.57 ± 0.95 ^c | 0.55 ^b | Synergism |
| | 0:1 | 72.54 ± 2.66 ^b | - | - |

Whereas DPPH; 2,2-diphenyl-1-picrylhydrazyl, ABTS; 2, 2' azino-bis (3- ethylbenzothiazoline-6-sulfonic acid) (ABTS), NO; nitric oxide, LPO; lipid peroxidation. EC₅₀: Effective concentration causing 50% scavenging activity; Results are expressed as mean value ± SD (n = 3). The dissimilar alphabets depict significant difference at $p \leq 0.05$ within the same column for a particular assay type. The type of interaction is classified based on combination index as follows: 0.1-0.3 (strong synergism), 0.3-0.7 (synergism), 0.7-0.85 (moderate synergism), 0.85-0.90 (synergism), 0.90-1.0 (nearly additive), 1.20-1.45 (moderate antagonism).

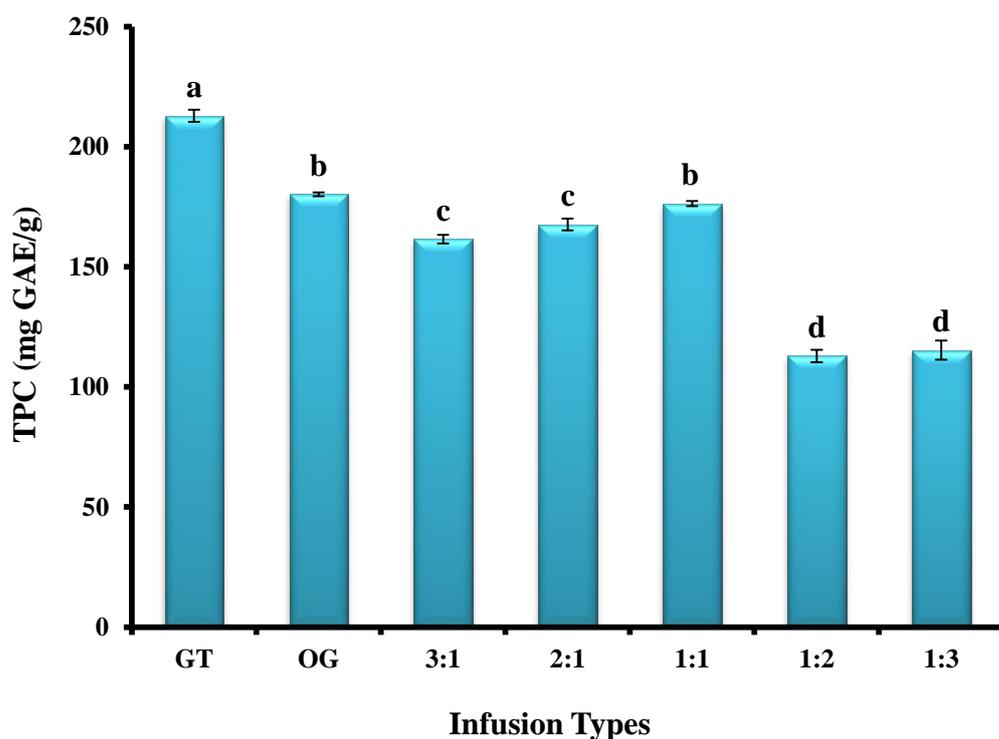
6.5.7 Total phenolic and flavonoid content of GT and OG mixture at five different ratios

6.5.7.1 Total phenolic content of GT and OG mixture at five different ratios

A linear calibration curve of gallic acid was prepared ranged from 20-120µg/ml as depicted in **Fig. 53a**. The TPC is denoted as gallic acid equivalents (GAE). The TPC of GT were found to be highest ($p \leq 0.05$) followed by OG \approx 1:1, 2:1 \approx 3:1 and 1:3 \approx 1:2, respectively, as shown in **Fig. 53b** and **Table 22**.



(a)



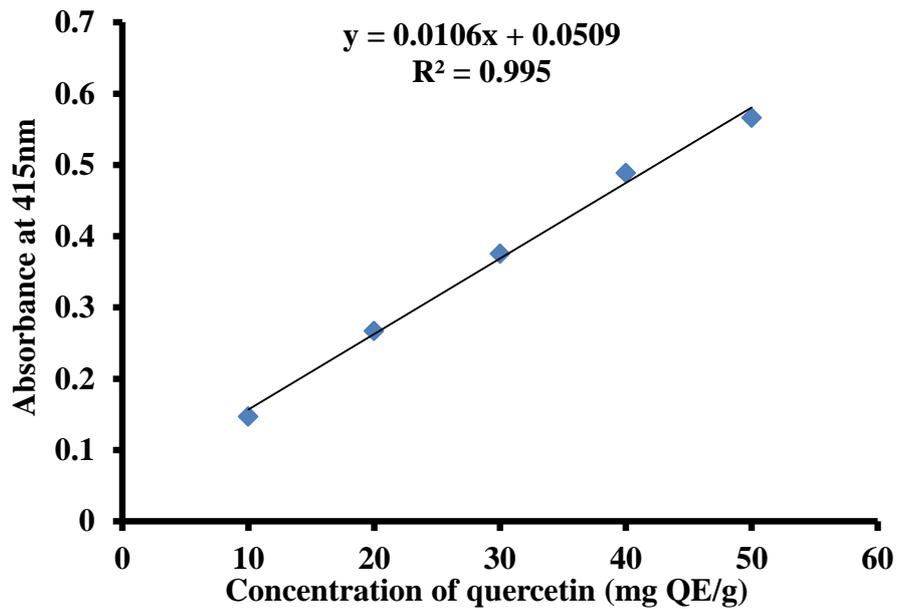
(b)

Fig. 53: (a) Standard calibration curve of gallic acid and (b) Total phenolic content (TPC) of GT, OG and their combination at five different ratios. The data is shown as MEAN \pm S.D for 3 independent tests (each with three replicates of each test point).

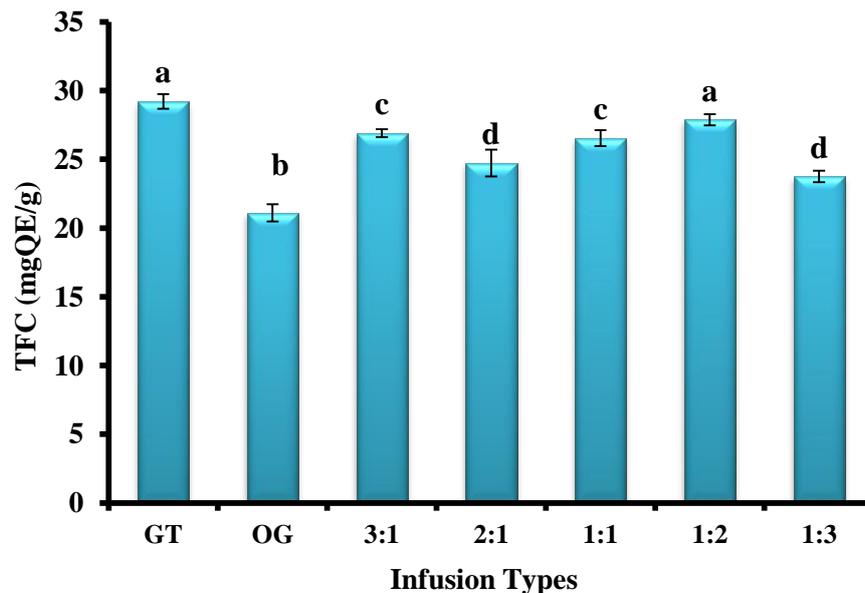
Different alphabets depicted significant difference at $p \leq 0.05$ among the TPC of different infusions.

6.5.7.2 Total favonoid content of GT and OG mixture at five different ratios

Flavonoids are type of polyphenolic compounds that demonstrate numerous biological activities. The TFC is represented as quercetin equivalents (QE). A standard curve of quercetin was prepared ranged from 10-50 $\mu\text{g}/\text{ml}$ as shown in **Fig. 54a**. The GT alone and binary combination (1:2) demonstrated similar ($p \geq 0.05$) but maximum TFC followed by 3:1 \approx 1:1, 2:1 \approx 1:3 and OG, respectively (**Fig. 54b**).



(a)



(b)

Fig. 54: (a) The standard calibration curve of quercetin and (b) Total flavonoid content (TFC) of GT, OG and their combination at five different ratios. The data is shown as MEAN \pm S.D for 3 independent tests (each with three replicates of each test point). Different alphabets depicted significant difference at $p \leq 0.05$ among the TFC of different infusions.

6.5.7.3 Comparison of theoretical and experimental value of TPC/TFC

To investigate the effect of green tea and *O. gratissimum* (GT+OG) combination at different proportions on the TPC and TFC, the difference between theoretical value and experimental value was evaluated. Theoretical value is calculated by the sum of phenolic or flavonoid content of green tea and *O. gratissimum* infusion separately, relative to their respective ratio in a binary mixture. A significant difference ($p \leq 0.05$) was observed between experimental and theoretical values of TPC at different ratios for GT+OG combination but TFC were found to be similar for all ratios except for 2:1 and 1:2, where minor differences were observed as displayed in **Table 22**.

Table 22: Total phenolic and flavonoid content of green tea and *O. gratissimum* combination at five different ratios.

| Ratio (GT:OG) | Theoretical value of TPC (mg GAE/g) | Experimental value of TPC (mg GAE/g) | Theoretical value of TFC (mg QE/g) | Experimental value of TFC (mg QE/g) |
|---------------|-------------------------------------|--------------------------------------|------------------------------------|-------------------------------------|
| 1:0 | - | 212.91 \pm 2.52 ^a | - | 29.21 \pm 0.54 ^a |
| 3:1 | 204.73 \pm 1.85* | 161.51 \pm 2.03 ^c | 27.18 \pm 0.52 | 26.90 \pm 0.30 ^b |
| 2:1 | 202 \pm 1.63* | 167.58 \pm 2.44 ^c | 26.51 \pm 0.52* | 24.73 \pm 0.97 ^c |
| 1:1 | 196.54 \pm 1.22* | 176.32 \pm 1.11 ^b | 25.16 \pm 0.53 | 26.54 \pm 0.58 ^b |
| 1:2 | 191.54 \pm 1.22* | 112.91 \pm 2.60 ^d | 23.81 \pm 0.56* | 27.88 \pm 0.41 ^b |
| 1:3 | 188.36 \pm 0.73* | 115.28 \pm 4 ^d | 23.13 \pm 0.57 | 23.75 \pm 0.42 ^c |
| 0:1 | - | 180.17 \pm 0.76 ^b | - | 21.11 \pm 0.63 ^d |

The data is represented as MEAN \pm S.D, n=3. The asterisk (*) denotes a significant difference ($p \leq 0.05$) between theoretical value and experimental value of TPC and TFC of GT and OG combination at a particular ratio, respectively. Different letters indicate significant difference in experimental value of TPC and TFC at different ratios. Total phenolic content (TPC), Total flavonoid content (TFC), GAE (gallic acid equivalents) and QE (quercetin equivalents).

6.5.7.4 Correlation between total phenolic/flavonoid content and antioxidant activities

The coefficient of correlation (r) between TPC/TFC and antioxidant activities (DPPH, ABTS, NO, LPO and Anti-haemolysis) was also investigated for individual infusions (GT and OG) and their combination. A very high negative correlation was detected between TPC/TFC and antioxidant activities (TPC/TFC-DPPH; $r = -0.997/-0.987$, TPC/TFC-ABTS; $r = -0.995/-0.994$, TPC/TFC-NO; $r = -0.982/-0.991$, TPC/TFC-LPO; $r = -0.950/-0.996$ and TPC/TFC-Anti-haemolysis; $r = -0.980/-0.989$) for individual infusions (GT and OG), whereas strength of correlation was high to moderate negative in GT+OG combination at different ratios (TPC – DPPH; $r = -0.890$, TPC-ABTS; $r = -0.893$; TPC-NO; $r = -0.670$, TPC-LPO; $R^2 = -0.909$ and TPC – Anti-haemolysis; $r = -0.765$). However, a negligible correlation was found between TFC and antioxidant potential of GT + OG combination (TFC - DPPH; $r = 0.245$, TFC – ABTS; $r = -0.053$, TFC – NO; $r = 0.117$, TFC – LPO; $r = -0.082$ and TFC – Anti-haemolysis; $r = 0.077$) as displayed in **Table 23**.

Table 23: Analysis of correlation coefficient (r) between total phenolic/ flavonoid content and antioxidant activities of individual and green tea and *O. gratissimum* combination (GT+OG) at different ratios.

| Antioxidant assays | TPC/TFC of all infusions | Correlation coefficient (r) of individual samples | Type of correlation | Correlation coefficient (r) of GT and OG combination at different ratios | Type of correlation |
|--------------------|--------------------------|---|---------------------|--|---------------------|
| DPPH | TPC | -0.997 | Very high (-ve) | -0.890 | High (-ve) |
| | TFC | -0.987 | Very high (-ve) | 0.245 | Negligible |
| ABTS | TPC | -0.995 | Very high (-ve) | -0.893 | High (-ve) |
| | TFC | -0.994 | Very high (-ve) | -0.053 | Negligible |
| NO | TPC | -0.982 | Very high (-ve) | -0.670 | Moderate (-ve) |
| | TFC | -0.991 | Very high (-ve) | 0.117 | Negligible |
| LPO | TPC | -0.950 | Very high (-ve) | -0.909 | High (-ve) |
| | TFC | -0.966 | Very high (-ve) | -0.082 | Negligible |
| Haemolysis | TPC | -0.980 | Very high (-ve) | -0.765 | High (-ve) |
| | TFC | -0.989 | Very high (-ve) | 0.077 | Negligible |

Whereas , 2, 2-diphenyl-1-picrylhydrazyl (DPPH), [(2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] (ABTS), nitric oxide (NO), lipid peroxidation (LPO), +ve (positive), -ve (negative), Total phenolic content (TPC) and Total flavonoid content (TFC).

6.5.8 FTIR analysis of GT and OG combination at different ratios

6.5.8.1 Green tea (GT)

The FTIR spectra of green tea as given in **Fig. 55** demonstrated a peak at 3747.81 cm^{-1} may assigned OH stretching of free alcohols. A wide peak at 3259.81 cm^{-1} in the region between $3000\text{-}3600\text{ cm}^{-1}$ indicated the overlapping of hydroxyl group of alcohols or phenols, CH stretching of alkene, arenes and NH stretching of amine groups. The absorption peaks at 2935.76 cm^{-1} exhibited (OH stretching of carboxylic acid and CH stretching of alkanes), 1699.34 cm^{-1} (C=O stretch of carboxylic acids), 1577.82 and 1527.67 cm^{-1} (NH bending of amide and C=C stretching aromatic compounds), 1492.95 and 1452.45 cm^{-1} (C=C stretching of aromatic groups and CH bending of alkanes), 1375.29 and 1325.14 cm^{-1} (CH bending of alkanes), 1259.59 cm^{-1} (CN stretching of amine), 1143.83 (CN stretching of amine), 1045.45 cm^{-1} (CO vibrations of phenols/alcohols/ethers/esters), 925.86 cm^{-1} (=C-H bending of alkenes), 813.99 and 773.48 cm^{-1} (=CH bending of alkenes and CH bending of aromatic compounds) as depicted in **Table 24**.

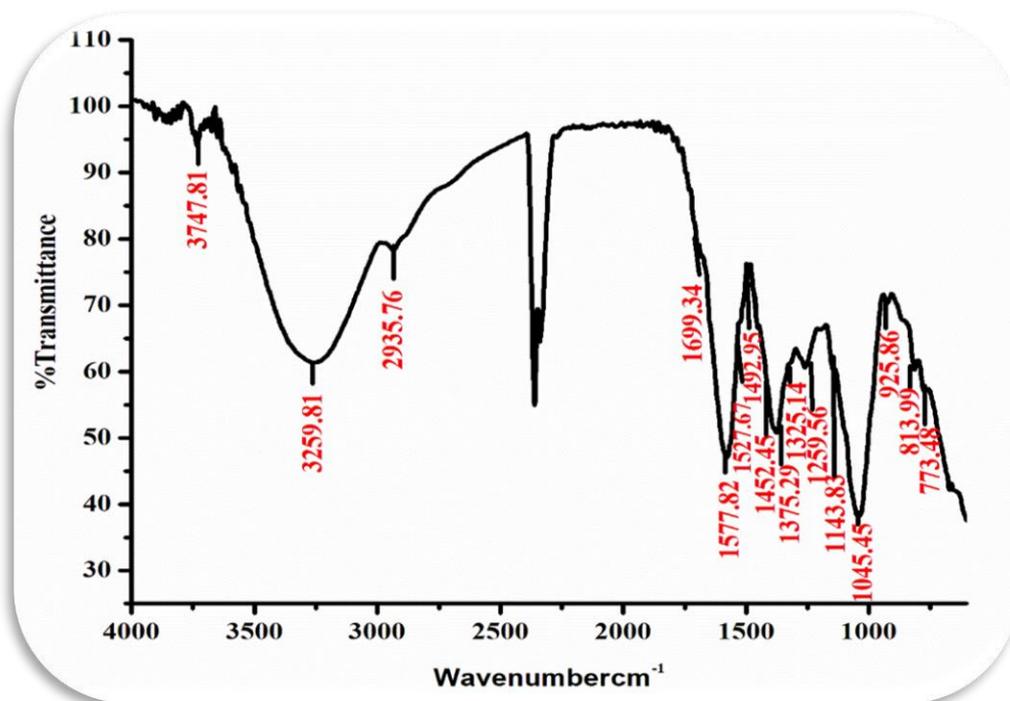


Fig.55: FTIR spectrum of green tea (2% GT) in the range of 600- 4000cm⁻¹.

6.5.8.2 *O. gratissimum* (OG)

The FTIR spectrum of dried *O. gratissimum* sample showed a number of peaks **Fig. 56**. A wide peak in range of 3000-3600 cm^{-1} displayed a band at 3261.74 cm^{-1} revealed the overlapping of hydroxyl group of alcohols or phenols, CH stretch of alkene, arenes and NH stretch of amine groups. The peaks at 2929.74 cm^{-1} assigned (OH stretching of carboxylic acid and CH stretching of alkanes), 1593.25 cm^{-1} (C=C stretching of aromatic compounds), 1373.36 cm^{-1} (CH bending of alkanes), 1238.34 cm^{-1} (CN stretching of amines and CO stretching carboxylic acids), 1143.83 cm^{-1} (CN stretching of amine), 1031.95 cm^{-1} (CO vibrations of ethers) and 815.92 cm^{-1} (= CH bending of alkenes and CH bending of aromatic compounds) as shown in **Table 24**.

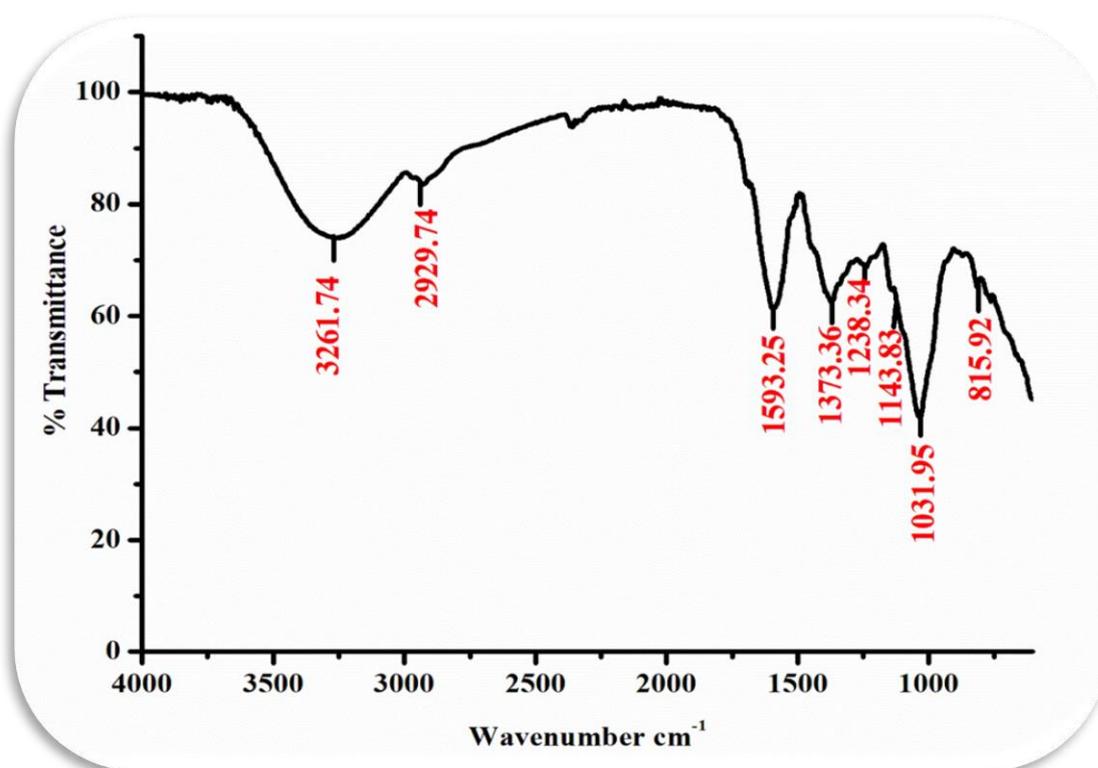


Fig. 56: FTIR spectrum of *O. gratissimum* (2% OG) in the range of 600- 4000 cm^{-1} .

6.5.8.3 Green tea and *O. gratissimum* combination (3:1) ratio

The FTIR spectra of binary combination (3:1) displayed a number of peaks (**Fig. 57**). Absorption peak 3738.17 cm^{-1} in the region between 3500-4000 cm^{-1} may due to OH stretching of free alcohols. A broad band obtained between 3000-3600 cm^{-1} gives a strong and broad peak at 3240.52 cm^{-1} indicated overlapping of hydroxyl groups of alcohols or

phenols, CH stretch of alkanes, aromatic structures and N-H stretch of amine groups. The absorption bands at 2933.83 assigned (OH stretching of carboxylic acid and CH stretching of alkanes), 1687.77 cm^{-1} (CO stretching of carboxylic acids), 1595.18 cm^{-1} (NH bending of amide and C=C stretching of aromatic groups), 1442.80 cm^{-1} (C=C stretching of aromatic groups and C-H bending of alkanes), 1375.29 cm^{-1} (CH bending of alkanes), 1247.99 cm^{-1} (CN stretch of amines and C-O vibrations of carboxylic acids), 1041.60 cm^{-1} (CO stretching of alcohols, phenols, ether and esters), 929.72 cm^{-1} assigned due to =CH bending of alkenes. The peak obtained at 813.99 (=CH bending of alkenes and CH bending of aromatic groups), 767.69 cm^{-1} (CH bending of aromatic groups, C-H bending of alkenes and C-Cl stretching of alkyl halides) and 671.25 cm^{-1} (C-Cl stretching stretching of alkyl halides) as depicted in Table 24.

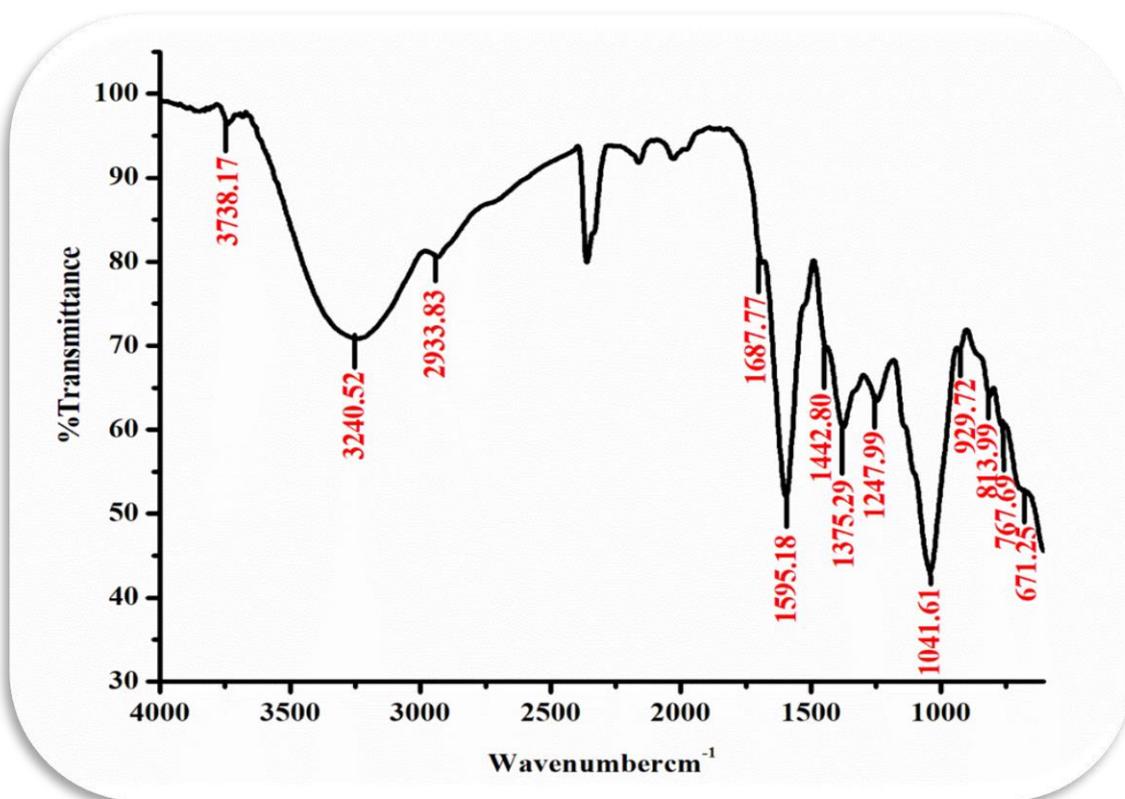


Fig. 57: FTIR spectrum of green tea and *O. gratissimum* combination at 3: 1 ratio in the range of 600- 4000 cm^{-1} .

6.5.8.4 Green tea and *O. gratissimum* combination (2:1) ratio

The peaks such as 3869.33 and 3738.17 cm^{-1} in the region between 3500-4000 cm^{-1} may due to OH vibrations of free alcohols as depicted in the FTIR spectrum of binary combination (2:1) **Fig. 58**. A strong and broad peak 3265.59 cm^{-1} in the region 3000-3600 cm^{-1} indicated

the overlapping of hydroxyl groups of alcohols or phenols, CH stretch of alkanes, aromatic structures and NH stretch of amine groups. The peaks obtained at 1622.19 cm^{-1} demonstrated N-H bending of amines and C=C stretching of alkenes; 1572.04 cm^{-1} , C=C stretch of aromatic groups and N-H bend of amide group; 1442.80 cm^{-1} , C=C stretching of aromatic groups and CH bending of alkanes; 1371.43 cm^{-1} , CH bending of alkane; 1217.12 cm^{-1} , CN stretching of amines and CO vibrations carboxylic acids; and 1051.24 cm^{-1} , CO stretching of alcohols/phenols/ether/esters) as displayed in **Table 24**.

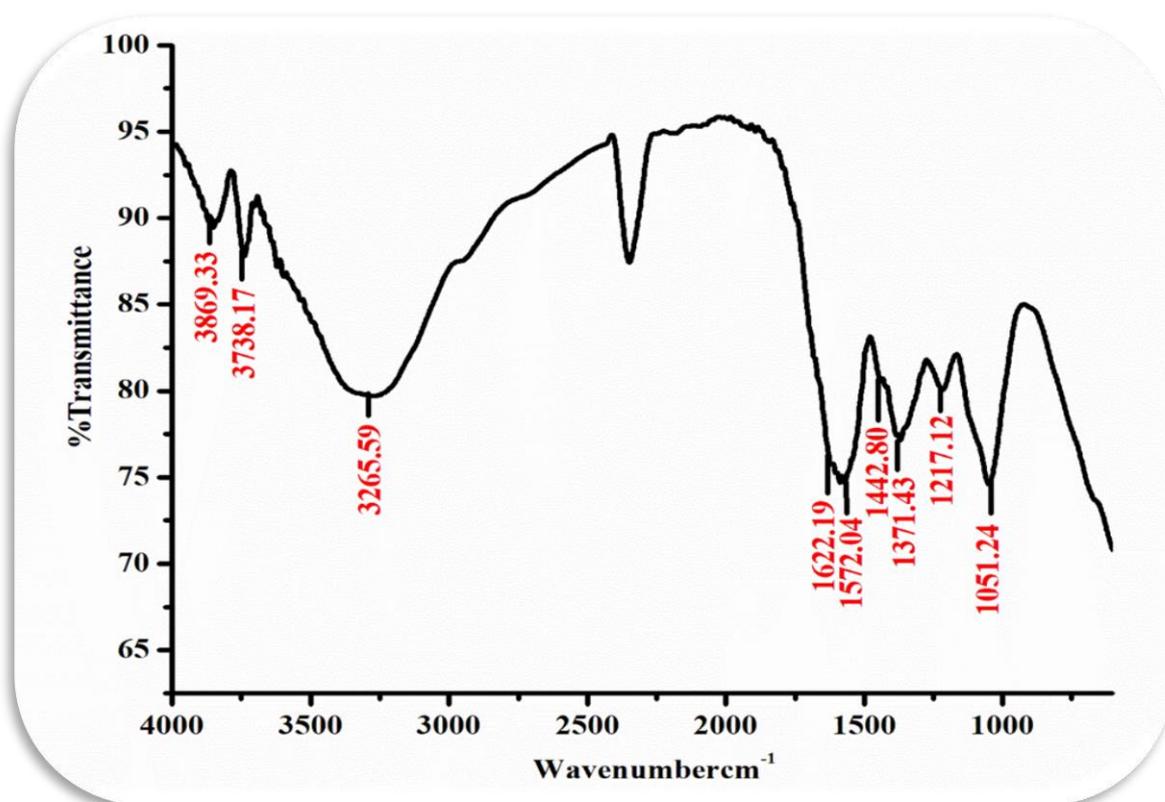


Fig. 58: FTIR spectrum of green tea and *O. gratissimum* combination at 2: 1 ratio in the range of $600\text{-}4000\text{cm}^{-1}$.

6.5.8.5 Green tea and *O. gratissimum* combination (1:1) ratio

The absorption peak at 3736.24 cm^{-1} may assigned (OH stretching vibrations) **Fig. 59**. The wide band of binary combination (1:1) obtained between $3000\text{-}3600\text{ cm}^{-1}$ gives sharp and broad peak at 3230.87 cm^{-1} demonstrated the overlapping of hydroxyl groups of alcohols or phenols, CH stretch of alkanes, aromatic structures and N-H stretch of amine groups. The absorption bands at 2933.8 demonstrated (O-H stretching of carboxylic acid and CH

stretching of alkanes), 1691.63 cm^{-1} (CO stretching of carboxylic acids), 1595.18 cm^{-1} (C=C stretching of aromatic groups and NH bend of amide group), 1525.74 cm^{-1} (C=C stretching of aromatic compounds), 1446.66 cm^{-1} (C=C stretching of aromatic groups and CH bending of alkanes), 1369.50 cm^{-1} (CH bending of alkanes), 1238.34 cm^{-1} (CN stretching of amines, C-O vibrations of ether and carboxylic acids), 1141.90 cm^{-1} (CN stretching of amine), 1039.67 cm^{-1} (CO stretching of alcohols/phenols/ether/esters), 925.86 cm^{-1} (=CH bending of alkenes), 813.99 cm^{-1} (=CH bending of alkenes, C-H bending of aromatic groups) and 657.75 cm^{-1} (C-Cl stretching of alkyl halides) as given in **Table 24**.

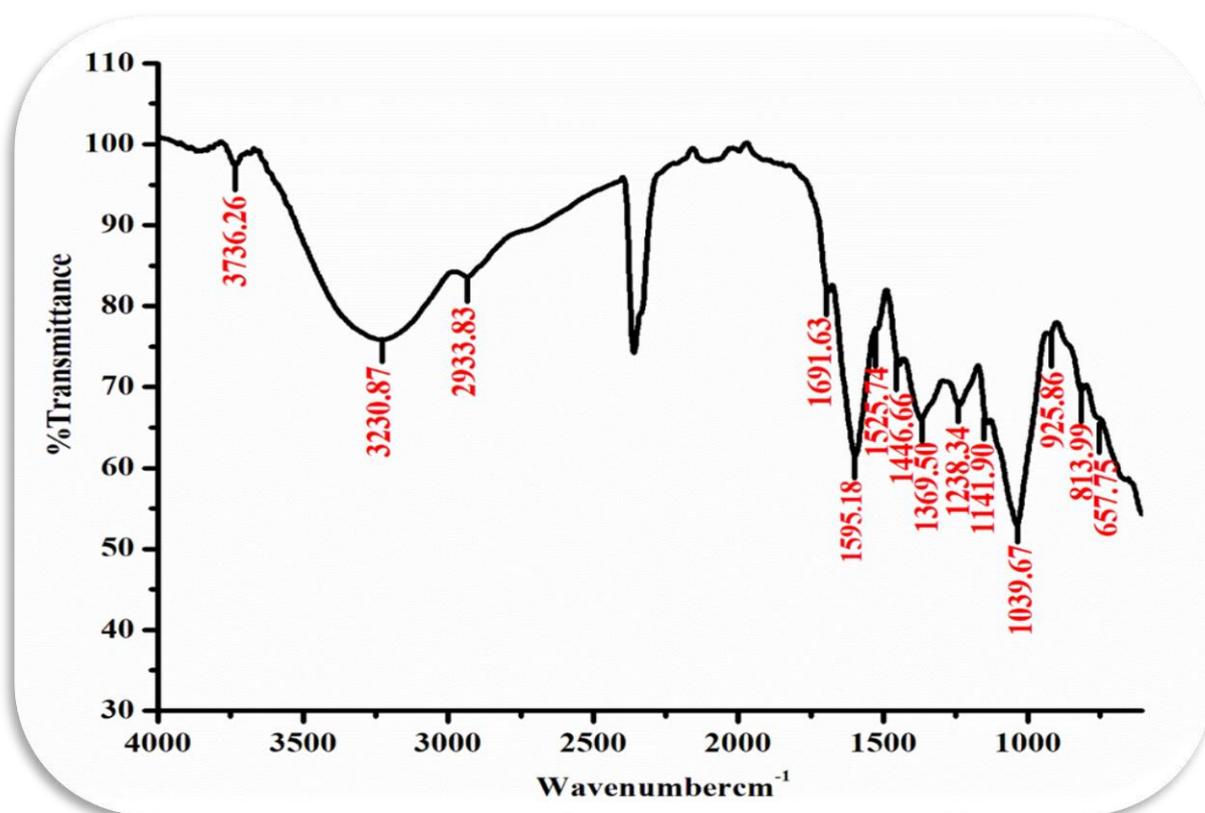


Fig. 59: FTIR spectrum of green tea and *O. gratissimum* combination at 1:1 ratio in the range of 600- 4000 cm^{-1} .

6.5.8.6 Green tea and *O. gratissimum* combination (1:2) ratio

The FTIR spectra of dried binary combination (1:2) showed number of peaks (**Fig. 60**). The band obtained at 3738.17 cm^{-1} may assigned due to OH stretching vibrations. The band between 3000-3600 cm^{-1} gives a strong and wide peak at 3228.95 cm^{-1} indicated the overlapping of hydroxyl groups of alcohols or phenols, CH stretch of alkanes, aromatic structures and NH stretch of amine groups. Different peaks at 2933.83 (OH stretching of carboxylic acid and CH stretching of alkanes), 1689.70 cm^{-1} (CO stretching of carboxylic

acids), 1595.18 cm^{-1} (C=C stretch of aromatic groups and NH bending of amide group), 1518.03 cm^{-1} (C=C stretch of aromatic compounds), 1446.66 cm^{-1} (CH bending of alkanes and C=C stretching of aromatic groups), 1371.43 cm^{-1} (CH bending of alkanes), 1238.34 cm^{-1} (CN stretching of amines and CO vibrations of carboxylic acids), 1141.90 cm^{-1} (CN stretching of amine), 1039.67 cm^{-1} (CO stretching of alcohols/phenols/esters/ether), 927.79 cm^{-1} (=CH bending of alkenes), 813.99 cm^{-1} (=CH bending of alkenes and C-H bending of aromatic groups) and 653.89 cm^{-1} (C-Cl stretch of alkyl halides) as shown in **Table 24**.

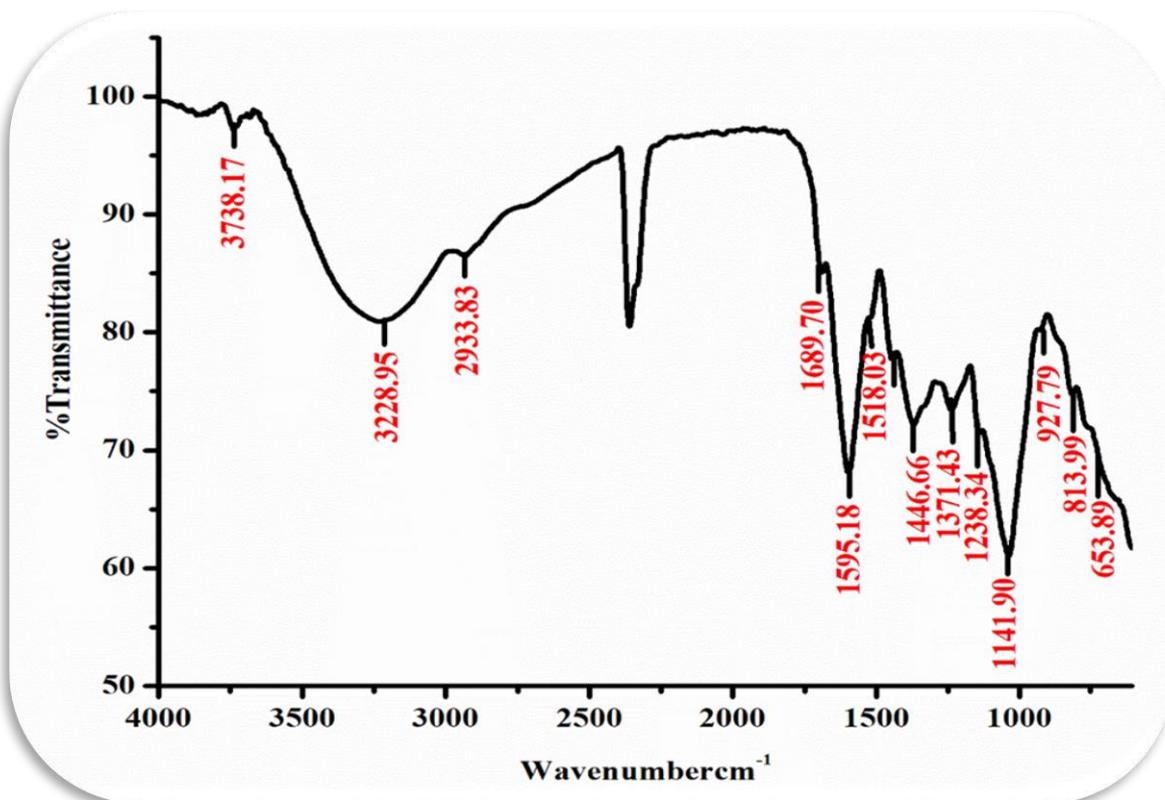


Fig. 60: FTIR spectrum of green tea and *O. gratissimum* combination at 1: 2 ratio in the range of 600- 4000 cm^{-1} .

6.5.8.7 Green tea and *O. gratissimum* combination (1:3) ratio

The spectrum of green tea binary mixture (1:3) displayed several peaks (**Fig. 61**). A peak at 3732.38 cm^{-1} assigned (OH stretching vibration) a strong and broad peak at 3246.31 cm^{-1} in the region between 3000-3600 cm^{-1} indicated the overlapping of hydroxyl groups of alcohols or phenols, CH stretch of alkanes, aromatic structures and NH stretch of amine groups. The absorption peaks at 2941.54 cm^{-1} (OH stretching of carboxylic acids and C-H stretching of alkanes), 1693.56 cm^{-1} (CO stretching of carboxylic acids), 1599.04 cm^{-1} (C=C stretch of aromatic groups, carboxylic acids, alkenes and N-H bending of amide group), 1454.38 cm^{-1}

(CH bending of alkanes and C=C stretching of aromatic compounds), 1369.50 cm^{-1} (CH bending of alkanes), 1242.20 cm^{-1} (CN stretch of amines and CO stretch of carboxylic acids), 1145.75 cm^{-1} (CN stretching of amine), 1062.81 cm^{-1} (CO stretching of alcohols/phenols/ester and ether), 927.79 cm^{-1} (=CH bending of alkenes), 813.99 cm^{-1} (=CH bending of alkenes and CH bending of aromatic groups) and 673.18 cm^{-1} (C-Cl stretch of alkyl halides) as displayed in Table 24.

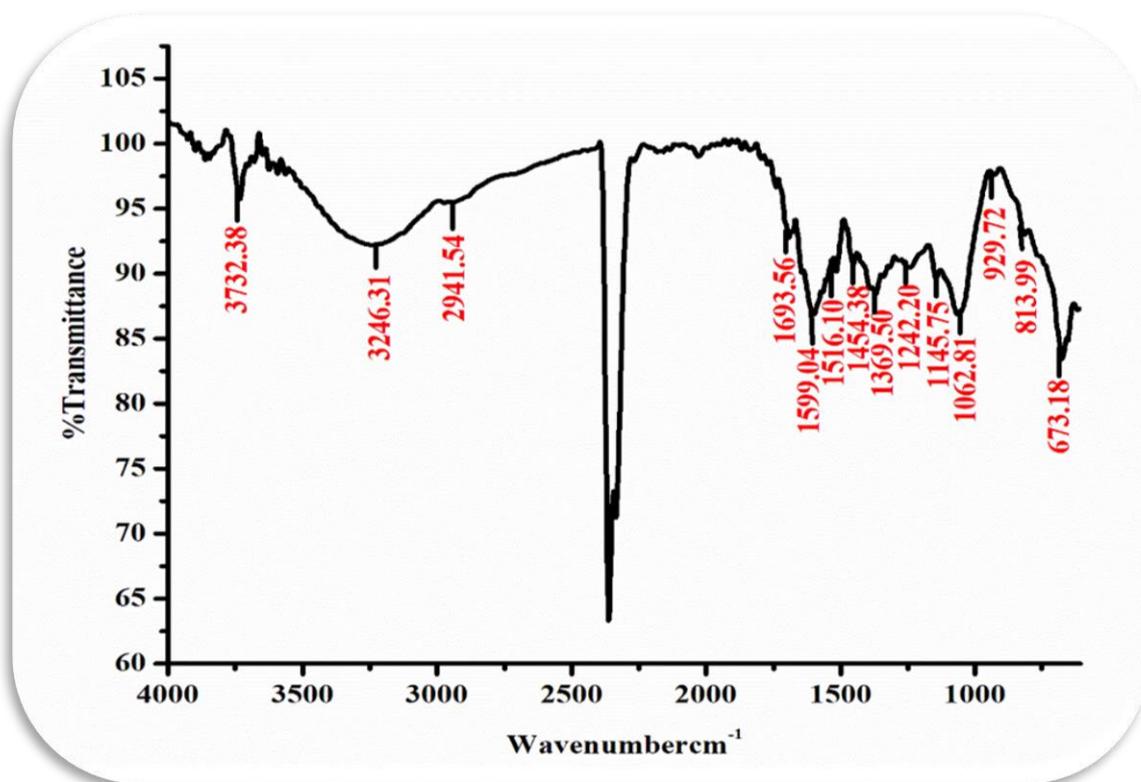


Fig. 61: FTIR spectrum of green tea and *O. gratissimum* combination at 1: 3 ratio in the range of 600- 4000cm⁻¹.

Table 24: The absorption spectrum of aqueous extracts of green tea, *Ocimum gratissimum* and their combination at five different ratios (3:1, 2:1, 1:1, 1:2 and 1:3)

| Functional groups | Absorption spectrum, frequency cm^{-1} | | | | | | | |
|-------------------|---|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| | Component (Peaks) | GT | OG | 3:1 | 2:1 | 1:1 | 1:2 | 1:3 |
| | Wave number (cm^{-1}) | | | | | | | |
| Alcohol/ Phenol | O-H (Stretch, H-bounded) (3200-3600) | 3259.81 | 3261.74 | 3240.52 | 3265.59 | 3230.87 | 3228.95 | 3246.31 |
| | O-H (Stretch, free) (3500-3800) | 3747.81 | - | 3738.17 | 3869.33, 3738.17 | 3736.24 | 3738.17 | 3732.38 |
| | C-O (Stretch) (970-1250) | 1045.45 | 1031.95 | 1041.60 | 1051.24 | 1039.67 | 1039.67, 1141.90 | 1062.81, 1145.75 |
| Carboxylic acids | C=O (Stretch) (1680-1710) | 1699.53 | - | 1687.77 | - | 1691.63 | 1689.70 | 1693.56 |
| | O-H (Stretch) (2500-3500) | 2935.95 | 2929.74 | 2933.83 | - | 2933.83 | 2933.83 | 2941.54 |
| | C-O (Stretch) (1210-1320) | 1259.56 | 1238.34 | 1247.99 | 1217.12 | 1238.34 | 1238.34 | 1242.20 |
| Alkane | C-H (Stretch) (2850-3000) | 2935.95 | 2929.74 | 2933.83 | - | 2933.83 | 2933.83 | - |
| | C-H(Bending) (1350-1480) | 1375.29, 1452.45 | 1373.36 | 1375.29, 1442.80 | 1371.43, 1442.80 | 1369.50, 1446.66 | 1371.43, 1446.66 | 1369.50, 1454.38 |
| Amine | N-H (Stretch) (2280-3380) | 3259.81 | 3261.74 | 3240.52 | 3265.59 | 3230.87 | 3228.95 | 3246.31 |
| | C-N (stretch) (1080-1360) | 1143.83, 1325.14 | 1143.83, 1238.34 | 1247.99 | 1217.12 | 1141.98, 1238.34 | 1141.98, 1238.34 | 1145.75, 1242.20 |
| Amide | N-H (Bending) (1550-1640) | 1527.67, 1577.83 | 1593.25 | 1595.18 | 1572.04, 1622.19 | 1595.18 | 1595.18 | 1599.04 |
| | C=C (Stretch) (1620-1680) | - | - | - | 1622.74 | - | - | - |

GT and OG represent green tea, *O. gratissimum*. FTIR spectral data interpretation: The characteristic bands of GT, OG and their combination at five different ratios were evaluated.

6.5.8.8 Comparison of FTIR spectra of GT, OG and their combination at different ratios

It was observed from the **Fig. 62**, that binary combinations (3:1, 2:1, 1:1, 1:2 and 1:3) revealed similar spectra as that of individual samples with difference in broadness and intensities of peaks and a slight shifting in the wavenumbers. It was clearly observed from the spectra of different samples that GT showed the strong and broad peak in the region of 3000-3600 cm^{-1} followed by 3:1, OG, 1:1, 2:1, 1:2, and 1:3, respectively. All the samples of green tea and *O. gratissimum* combination at five ratios demonstrated some common features. The GT and OG combination at different proportions have O-H peaks at 3240.52, 3265.59, 3230.87, 3228.95 and 3246.31 cm^{-1} , respectively. These peaks are having lower intensity than that of GT at 3259.81 cm^{-1} and higher than OG at 3261.74 cm^{-1} . The absorption peaks in 3000-3600 cm^{-1} region demonstrated a strong and broadband for all ratios.

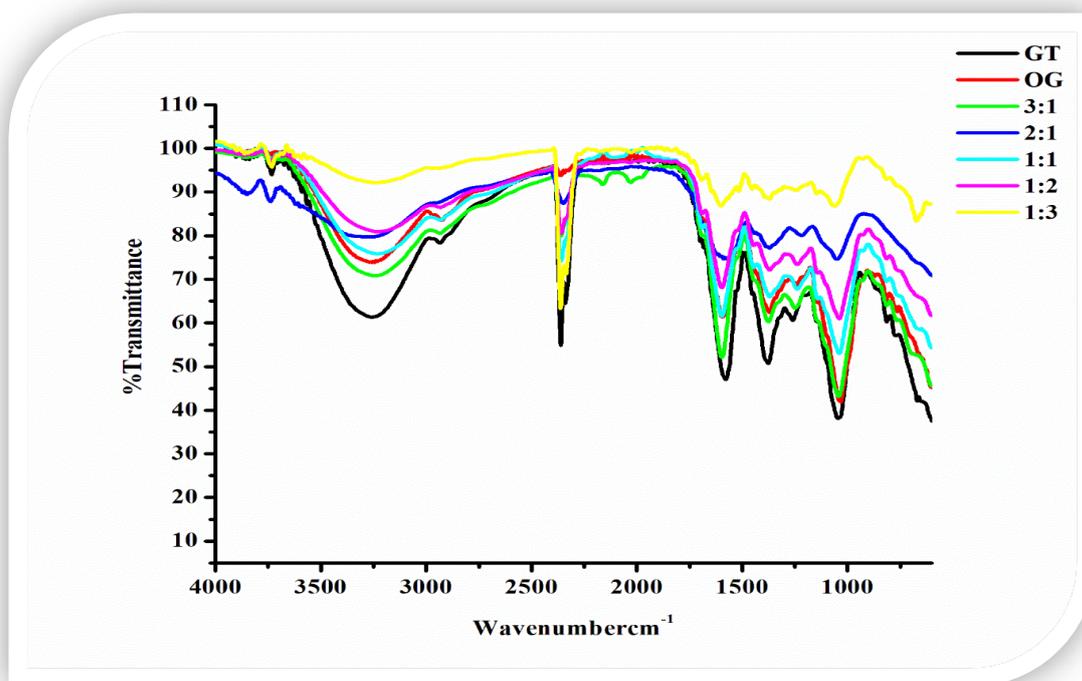


Fig. 62: Comparison of FTIR spectra of all GT and OG combination at different ratios with a scan range from 600-4000 cm^{-1}

6.5.8.9 Correlation analysis between FTIR and Total phenolic content/antioxidant activities

There was a high negative correlation ($r = -0.855$) between TPC and peak intensity in the IR spectrum range between 3000-3600 cm^{-1} , but negligible correlation between EC_{50} and

intensity of the peak for antioxidant tests [(DPPH, $r = 0.163$), (ABTS, $r = -0.052$), (NO, $r = -0.090$), (LPO, $r = -0.280$) and (haemolysis, $r = -0.146$)] as given in **Table 25**.

Table 25: A correlation analysis between intensity of absorbance peak (3228-3266 cm^{-1}) and total phenolic content or antioxidant activity of green tea and *O. gratissimum* combination at different ratios.

| Parameter | Correlation coefficient (r) | Type of correlation |
|------------|-----------------------------|---------------------|
| TPC | -0.855 | High (-ve) |
| DPPH | 0.163 | Negligible |
| ABTS | -0.052 | Negligible |
| NO | -0.090 | Negligible |
| LPO | -0.280 | Negligible |
| Haemolysis | -0.146 | Negligible |

Whereas, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), [(2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] (ABTS), nitric oxide (NO), lipid peroxidation (LPO), +ve (positive), -ve (negative), Total phenolic content (TPC), Total flavonoid content (TFC) and Fourier transform infrared spectrum (FTIR)

6.5.9 UV-VIS for GT and OG combination at different ratios

The UV-VIS absorbance spectra of different aqueous extracts such as green tea (GT), *O.gratissimum* and binary combinations (3:1, 2:1, 1:1, 1:2 and 1:3) were evaluated at wavelength (λ) 200nm to 600nm at room temperature **Fig. 63**. The absorbance peak of the GT showed highest absorbance (1.758) followed by 3:1 (1.266) and 2:1 (1.149) samples at the wavelength (λ_{max}) 270 nm whereas 1:2, 1:3 and OG samples illustrated high absorbance 1.086, 0.993 and 0.381, respectively at 271nm. However, 1:1 ratio demonstrated high absorbance (1.159) at 272nm. Green tea demonstrated the highest absorption peak followed by 3:1, 1:1, 2:1, 1:2, 1:3 and the least absorbance was noticed for *O. gratissimum*. The spectral intensity of green tea, and GT and OG combination at various ratios drops near to base at wavelength greater than 400nm.

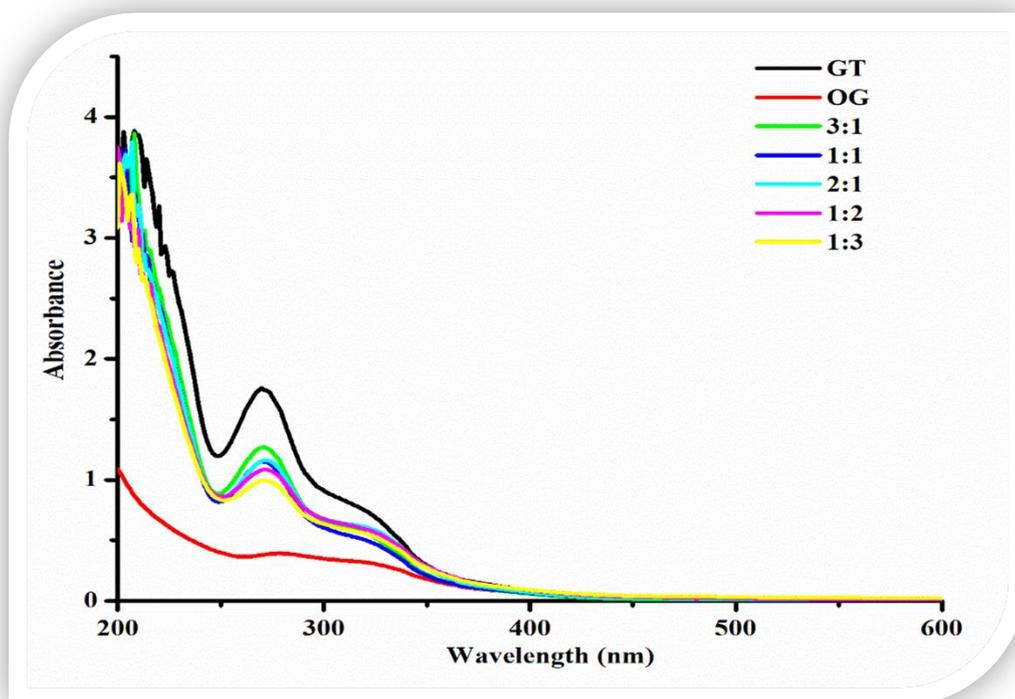


Fig. 63: UV–VIS spectra of GT, OG and their combination at different ratios

6.5.9.1 Correlation analysis between ultraviolet region absorption peak and antioxidant activities of green tea combination at various ratios

A moderate to high negative correlation was observed between absorbance (272nm) and EC₅₀ of antioxidant assays [LPO (r = -0.590), Haemolysis (r = -0.677), NO (r = -0.775), ABTS (r = -0.819) and DPPH (r = - 0.853)], but a low positive correlation (r = 0.304) was reported between TPC and absorbance of the different samples given in **Table 26**.

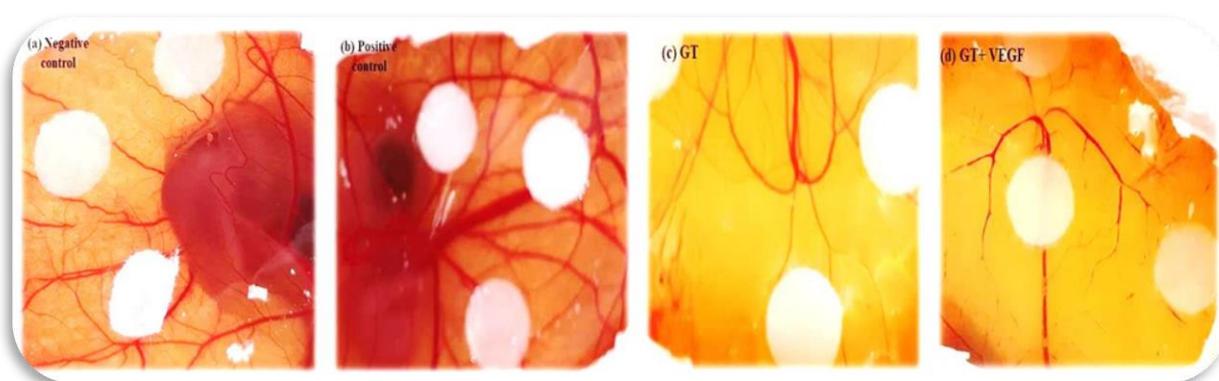
Table 26: A correlation analysis between ultraviolet region absorbance peak (272nm) and total phenolic content or antioxidant activities of green tea combinations at different ratios.

| Parameter | Correlation coefficient (r) | Type of correlation |
|-------------------|------------------------------------|----------------------------|
| TPC | 0.304 | Low (+ve) |
| DPPH | -0.853 | High (-ve) |
| ABTS | -0.819 | High (-ve) |
| NO | -0.775 | High (-ve) |
| LPO | -0.590 | Moderate (-ve) |
| Haemolysis | -0.677 | Moderate (-ve) |

Whereas , 2, 2-diphenyl-1-picrylhydrazyl (DPPH), [(2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] (ABTS), nitric oxide (NO), lipid peroxidation (LPO), +ve (positive), -ve (negative) and Total phenolic content (TPC)

6.6 *In vivo* anti-angiogenic activity of individual infusions and green tea combinations

The representative optical images showed difference in the development of microvascular network among different sample treated groups. The vascular endothelial growth factor (VEGF) treated group (positive control) promoted high capillary growth with various branches **Fig. 64b** as compared to normal control **Fig.64a**. In contrast, VEGF plus different infusion treated groups exhibited partial microvessels formation, the reduction was observed in the new blood capillaries and their branching without affecting the mature blood vessels as noted in **Fig. 64c-t**. The average number of blood vessels lasting in the CAM after treatment of various aqueous infusions, VEGF and NaCl are given in **Fig. 65** and **Table 27**. All the aqueous infusions such as (GT, OG, CC, CF, HR, GT+OG, GT+CC, GT+CF and GT+HR) demonstrated an inhibition in angiogenesis in CAM model. At some extent the blood vessel formation was reduced by all infusions at a concentration of 5µg/ml. However, the higher but similar ($p \geq 0.05$) anti-angiogenic effect was displayed by GT and GT+OG infusions. The CAM assay was also performed under the induction of angiogenesis by VEGF (20µg/embryo) with addition of infusions GT, OG, CC, CF, HR, GT+OG, GT+CC, GT+CF and GT+HR, respectively, revealed a decrease ($p \leq 0.05$) in total vessel length compared to positive control. A significant difference was also noticed in blood vessel formation between infusion treated alone and both VEGF and infusion treated groups ($p \leq 0.05$). The GT and GT+OG sample showed higher reduction in the total vessel length. The individual samples such as OG, CC, CF and HR demonstrated similar activity as that of green tea combinations.



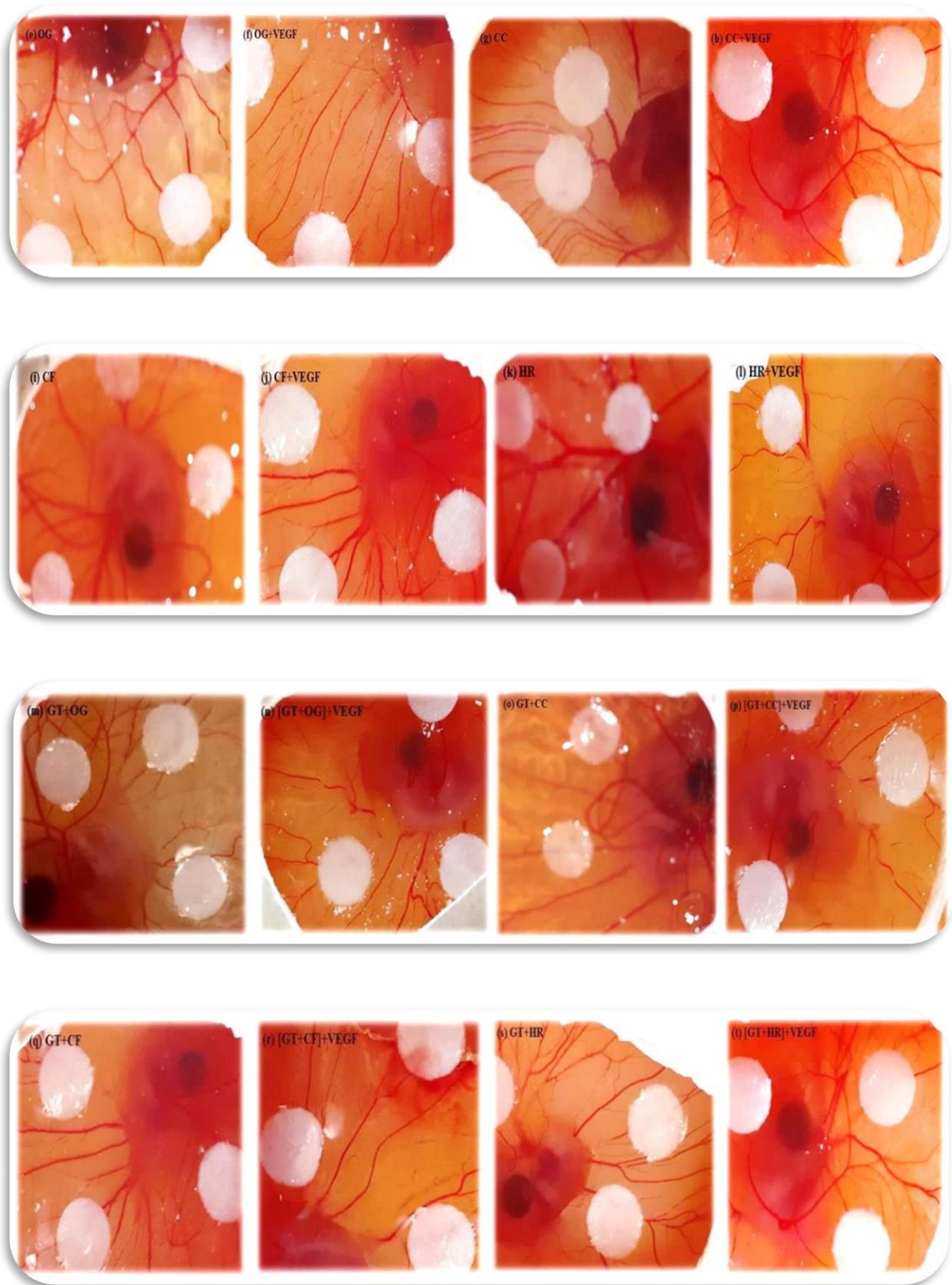


Fig. 64: The chick embryo chorioallantoic membrane (CAM) models of 8-day-old treated as: (a) 0.9% NaCl alone; (b) vascular endothelial growth factor (VEGF) (c) aqueous infusion of GT (d) aqueous infusion of GT plus VEGF (e) aqueous infusion of

OG, (f) aqueous infusion of OG plus VEGF (g) aqueous infusion of CC (h) aqueous infusion of CC plus VEGF (i) aqueous infusion of CF (j) aqueous infusion of CF plus VEGF, (k) aqueous infusion of HR, (l) aqueous infusion of HR plus VEGF, (m) aqueous infusion of GT+OG, (n) aqueous infusion of GT+ OG plus VEGF, (o) aqueous infusion of GT+CC, (p) aqueous infusion of GT+CC plus VEGF,(q) aqueous infusion of GT+CF, (r) aqueous infusion of GT+CF plus VEGF, (s) aqueous infusion of GT+HR and (t) aqueous infusion of GT+HR plus VEGF.

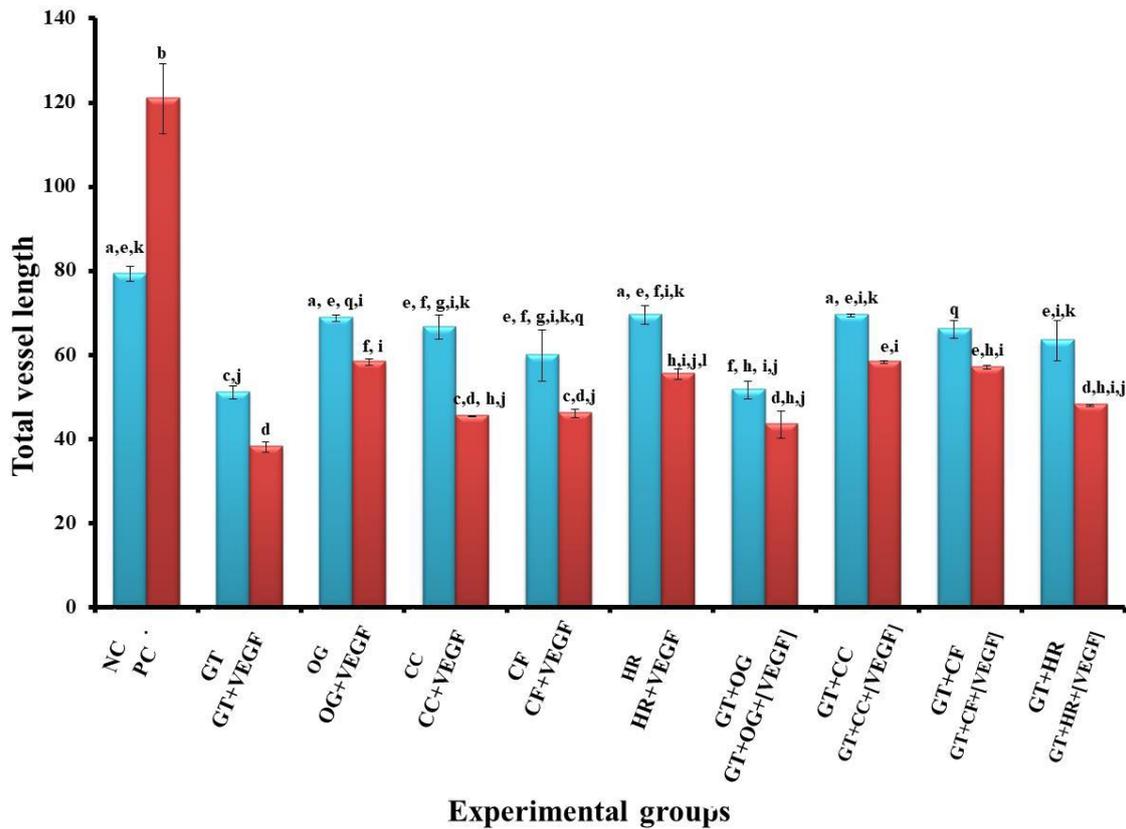


Fig. 65: Effect of aqueous extracts on total blood vessel length in chick embryo assay. The dissimilar alphabets displayed a significant difference among the total vessel length in different experimental groups separately ($p \leq 0.05$). Values represented mean \pm SD (n=3).

Table 27: The total vessel length of different groups treated with various infusions

| Groups | | Total vessel length | Groups | | Total vessel length |
|--------|---------|-----------------------------------|--------|--------------|---------------------------------|
| 1. | NC | 79.26 ± 1.81 ^{a,e,k} | 2. | PC | 120.85 ± 8.28 ^b |
| 3. | GT | 51.06 ± 1.48 ^{c,j} | 4. | GT+VEGF | 38.08 ± 1.27 ^d |
| 5. | OG | 68.69 ± 0.81 ^{a,e,i} | 6. | OG+VEGF | 58.33 ± 0.79 ^{f,l} |
| 7. | CC | 66.53 ± 2.88 ^{a,f,g,i,k} | 8. | CC+VEGF | 45.47 ± 0.09 ^{c,d,h,j} |
| 9. | CF | 59.62 ± 6.12 ^{e,f,g,i,k} | 10. | CF+VEGF | 46.10 ± 0.94 ^{c,d,j} |
| 11. | HR | 69.55 ± 2.17 ^{a,e,f,i,k} | 12. | HR+VEGF | 55.43 ± 1.26 ^{h,i,j,l} |
| 13. | [GT+OG] | 51.70 ± 2.07 ^{f,h,i,j} | 14. | [GT+OG]+VEGF | 43.39 ± 3.20 ^{d,h,k} |
| 15. | [GT+CC] | 69.41 ± 0.39 ^{a,e,i,k} | 16. | [GT+CC]+VEGF | 58.32 ± 0.25 ^{e,l} |
| 17. | [GT+CF] | 66.11 ± 2.05 ^{e,i,k} | 18. | [GT+CF]+VEGF | 57.00 ± 0.46 ^{e,h,l} |
| 19. | [GT+HR] | 63.41 ± 4.68 ^{e,i,k} | 20. | [GT+HR]+VEGF | 48.05 ± 0.24 ^{d,h,i,j} |

Positive control (PC, VEGF), negative control (NC, NaCl), green tea (GT), *Ocimum gratissimum* (OG), *Cymbopogon citratus* (CC), *Cymbopogon flexuosus* (CF), *Hibiscus rosa-sinensis* (HR) and the vascular endothelial growth factor (VEGF). Different letters showed significant difference in the vessel length of different experimental groups.

6.7 Antioncogenic and cancer preventive potential of green tea, *O. gratissimum* and their combination

The GT and OG combination (1:1) was evaluated for anti-oncogenic activity because this binary combination demonstrated higher antioxidant capacity by applying several cell free and *ex-vivo* models.

6.7.1 Determination of cytotoxicity by applying Sulforhodamine B (SRB) test

The inhibition of human lung cancer cell line (A549) growth was checked by SRB assay after 48 hours of treatment with GT, OG, GT+OG, and Adriamycin (ADR) a known anticancer drug at various doses (10, 20, 40 and 80µg/ml). ADR was most effective in inhibition of cell viability. All infusions showed cytotoxic capability as compared to untreated control cells. GT and GT+OG displayed comparable cytotoxic ability in a dose independent manner **Fig. 66** and **Table 28**. These infusions illustrated greater inhibition of

cell growth as compared to OG ($p \leq 0.05$) at low concentration whereas no difference was observed at higher doses ($p \geq 0.05$). The antioncogenic potential of infusions was examined on A549 lung cancer cell line by determining inhibition of cell growth employing SRB assay.

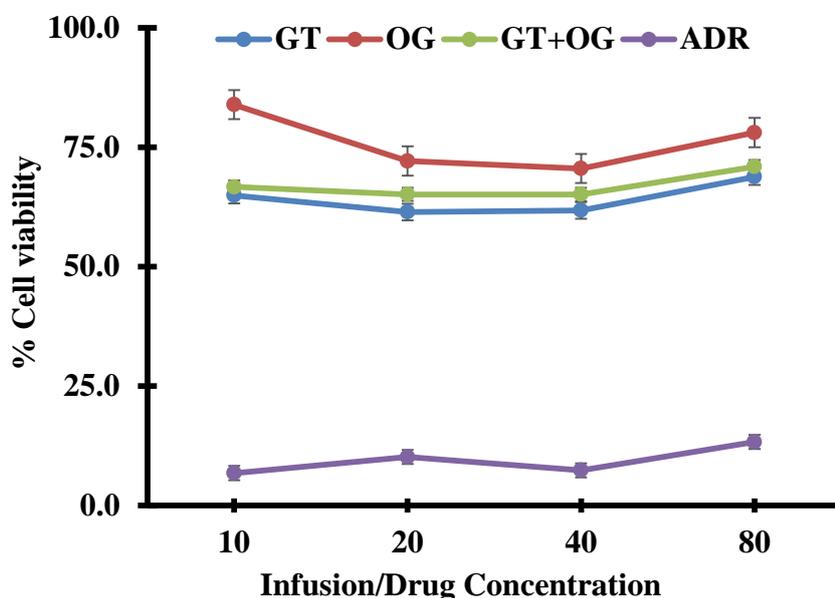


Fig. 66: Effect of infusions and Adriamycin on A549 lung cancer cell proliferation. The data is shown as MEAN \pm S.D of two similar experiments each performed in triplicate. Green tea (GT), *Ocimum gratissimum* (OG) and their combination (GT+OG), and Adriamycin (ADR).

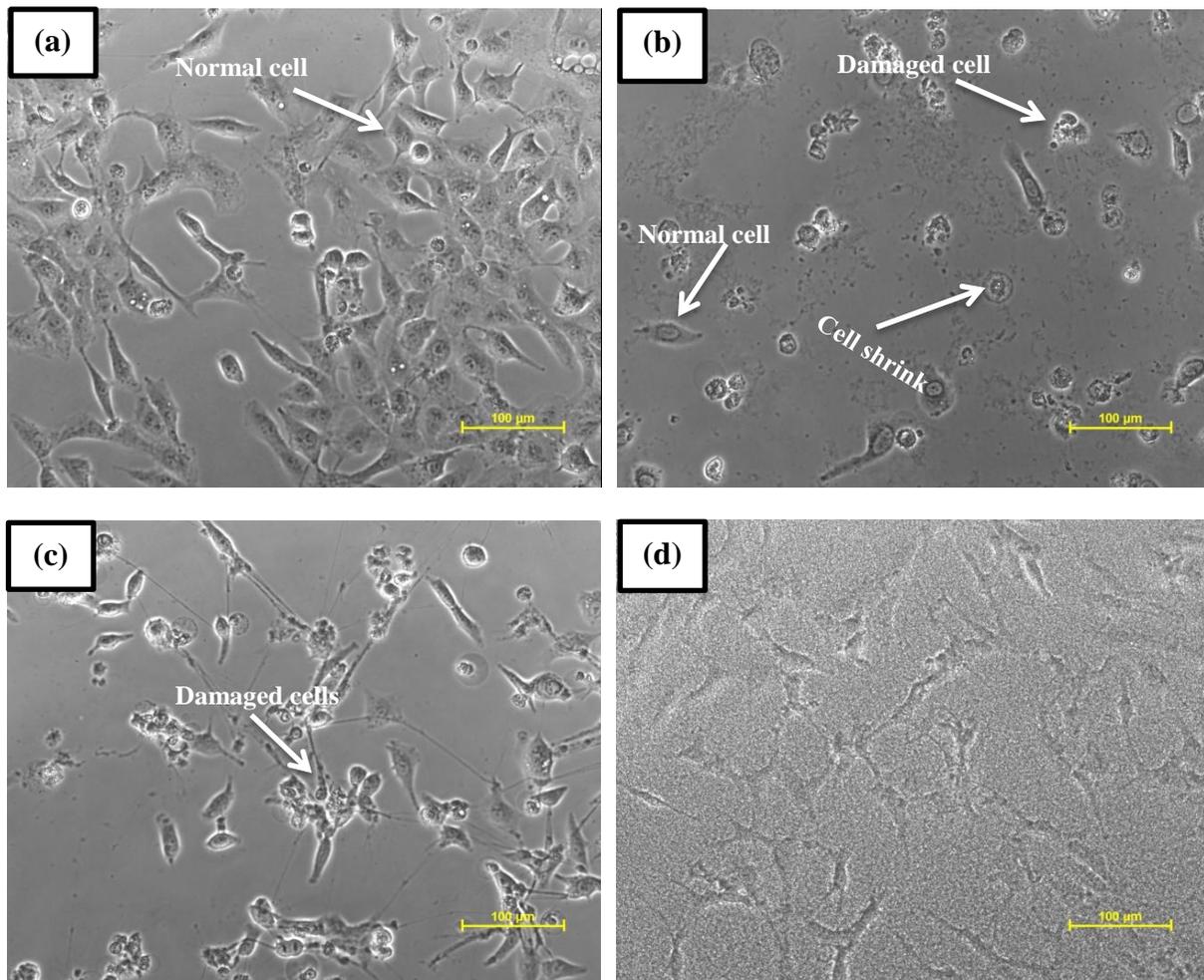
Table 28: Cell viability of A549 lung cancer cells by various aqueous extracts at different concentrations

| S.No | Samples | Cell viability % | | | |
|------|---------|---|--------------------|--------------------|--------------------|
| | | Sample concentration ($\mu\text{g/ml}$) | | | |
| | | 10 | 20 | 40 | 80 |
| 1. | GT | 65.00 ^a | 61.50 ^a | 61.80 ^a | 68.80 ^a |
| 2. | OG | 83.95 ^b | 72.15 ^c | 70.55 ^c | 78.10 ^c |
| 3. | GT+OG | 66.75 ^d | 65.15 ^d | 65.15 ^d | 70.95 ^d |
| 4. | ADR | 6.80 ^e | 10.15 ^e | 7.40 ^e | 13.30 |

Green tea (GT), *O. gratissimum* (OG) and Adriamycin (ADR)

6.7.2 Cell morphology

The GT, OG, GT+OG and ADR induced changes in the morphology of A549 cells after 48 hours of treatment at the 80 μ g/ml of concentration were observed and photographed by using a phase contrast microscope as displayed in **Fig. 67a-e**. The features of cell death, such as cell shrinkage and detachment of cells from substratum was observed upon treatment of ADR, GT, GT+OG and less in OG compared to control cells. The alterations in perimeter, area and the roundness of the cell were noticed in the treatment groups.



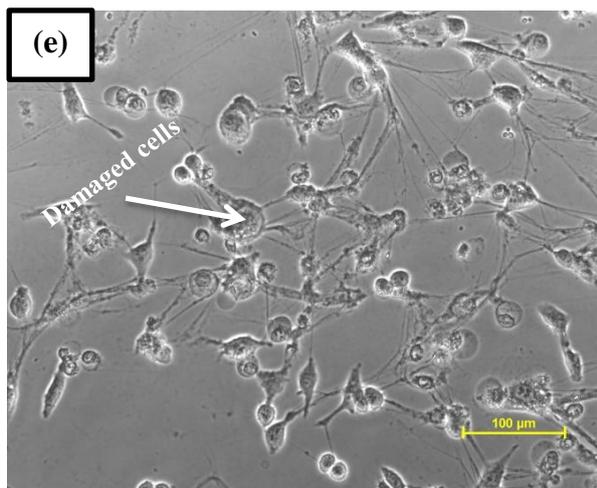


Fig. 67: Morphological analysis (a) Control (b) Adriamycin (c) GT (d) OG and (e) GT+OG by using a phase contrast microscope. All pictures are typical of three independent experiments taken at higher concentration (80μg/ml), each performed under identical conditions. Scale bar is 100μm.

6.8 Effect of green tea, *O. gratissimum* and their combination against benzo(a)pyrene induced lung tumorigenesis

6.8.1 Body status, food and infusion intake

The initial body weight was found to be similar in all groups of mice. The body weight of all animals increases till 6th week of experiment after that body weight remains constant and start decreasing in all groups at 16th week after that it remains constant as shown in **Fig. 68**. The water consumption and food intake were observed to be similar in all groups of animals **Table 29**. A wide variation was observed in fluid and food consumption within the same group. The final body weight or the relative lung weight was observed to be similar in all the groups of animals as shown in **Table 30**.

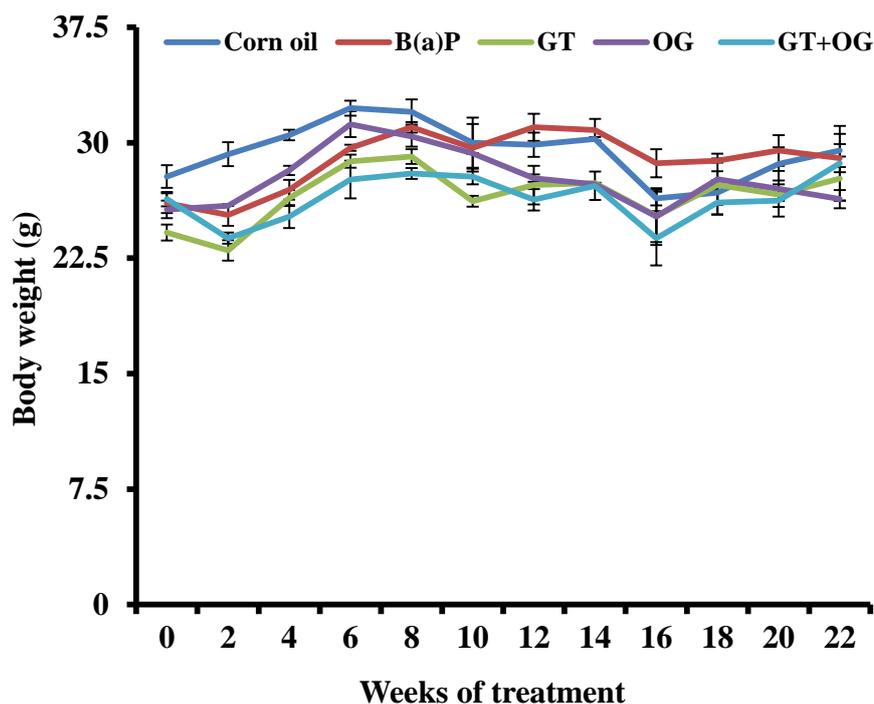


Fig. 68: Effect of B(a)P along with green tea, *O. gratissimum* and their combination on body weight of mice in various groups. The values represented mean \pm S.D for six mice in each group. Whereas, Group I: Corn oil, Group II: Benzo(a)pyrene [B(a)], Group III: Green tea (GT), Group IV: *O. gratissimum*(OG) and Group V: green tea and *O.gratissimum* (GT+OG) combination. Body weight is expressed in grams (g).

Table 29: The influence of oral administration of green tea, *O. gratissimum* and their combination on fluid and food consumption of different experimental groups.

| Groups | Fluid intake (ml/mouse/day) | Food consumption (g/mouse/day) |
|--------|-----------------------------|--------------------------------|
| I. | 16.72 | 19.35 |
| II. | 17.05 | 17.74 |
| III. | 11.90 | 15.47 |
| IV. | 13.58 | 13.28 |
| V. | 13.68 | 17.36 |

Values represented mean \pm S.D for six mice per group. Whereas, Group I (vehicle control), Group II (Benzo(a)pyrene), Group III (Green tea), Group IV (*O. gratissimum*) and Group V (green tea and *O. gratissimum* combination). Fluid and food consumption expressed as ml/mice/day and g/mice/day, respectively.

Table 30: Effect of B(a)P along with green tea, *O. gratissimum* and their combination on body and relative lung weight of various group of animals.

| Groups | Number of mice | Body weight (g) | Relative Lung Weight (mg/g BW) |
|-----------|----------------|-----------------|--------------------------------|
| Group I | 5 | 30.00 ± 1.00 | 7.66 ± 0.57 |
| Group II | 5 | 28.00 ± 0.81 | 6.98 ± 0.82 |
| Group III | 6 | 25.75 ± 4.03 | 8.08 ± 1.69 |
| Group IV | 5 | 26.20 ± 3.56 | 8.14 ± 1.34 |
| Group V | 5 | 27.25 ± 3.77 | 8.02 ± 1.69 |

Whereas Group I: vehicle control, Group II: Benzo(a)pyrene, Group III: Green tea, Group IV: *O. gratissimum* and Group V: green tea and *O. gratissimum* combination. Values represented mean ± S.D for six mice in each group. Both body weight and relative lung weight is expressed in grams (g and mg/g). BW (body weight).

6.8.2 Biochemical assays

6.8.2.1 DPPH scavenging assay

The plasma scavenging capacity against DPPH radical was observed to be declined in the B(a)P treated group (Gp. II) as compared to vehicle control group (Gp. I). The oral consumption of aqueous infusion of green tea (Gp. III), *O. gratissimum* (Gp. IV) and their combination (Gp. V) demonstrated higher plasma DPPH scavenging potential as compared to animals administered B[a]P ($p \leq 0.05$) as displayed in **Fig. 69**. The green tea and *O. gratissimum* combination (GT+OG) illustrated maximum scavenging activity followed by green tea (GT), and both treatments showed higher activity in comparison to vehicle control. However, *O. gratissimum* (OG) normalized the activity as that of Gp. I.

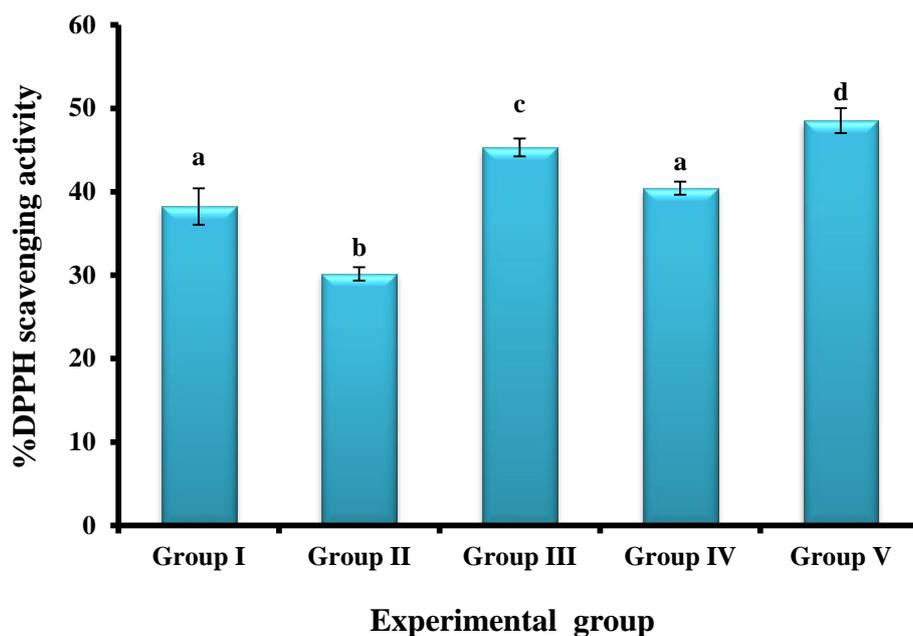


Fig. 69: Plasma DPPH scavenging activity of various experimental groups. Group I: Corn oil, Group II: Benzo(a)pyrene, Group III: Green tea, Group IV: *O. gratissimum* and Group V: green tea and *O.gratissimum* combination. Dissimilar alphabets demonstrated a difference ($p \leq 0.05$) among the activity of different experimental groups. Values represented the mean \pm S.D; n=3 and 2, 2-diphenyl-1-picrylhydrazyl (DPPH).

6.8.2.2 ABTS scavenging assay

Animals treated with benzo(a)pyrene (Gp. II) showed decreased level of plasma scavenging capacity against ABTS radical compared to vehicle control group (Gp. I). Oral consumption of GT (Gp. III), OG (Gp. IV) and GT+ OG (Gp. V) demonstrated an increased plasma ABTS radical scavenging activity. It was found that GT+OG exhibited highest plasma ABTS scavenging activity. However, GT and OG normalized the activity as that of Gp. I as shown in **Fig.70**.

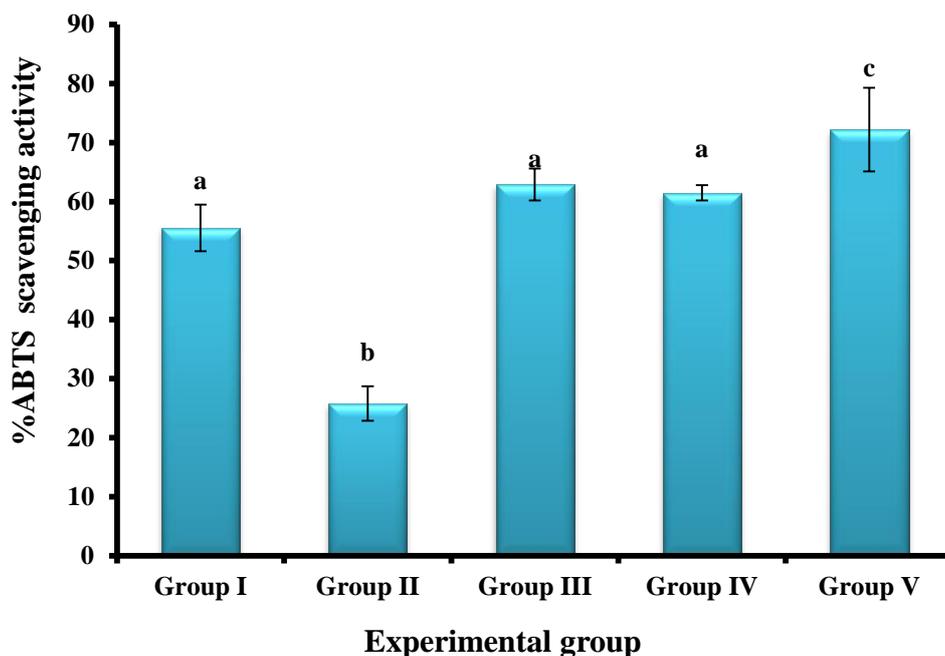


Fig. 70: Plasma ABTS scavenging activity of various experimental groups. Group I: Corn oil, Group II: Benzo(a)pyrene, Group III: Green tea, Group IV: *O. gratissimum* and Group V: green tea and *O. gratissimum* combination. Dissimilar alphabets demonstrated a difference ($p \leq 0.05$) among the activity of different experimental groups. Values represented the mean \pm S.D; n=3 and 2, 2' azino - bis (3-ethylbenzothiazoline-6- sulfonic acid (ABTS).

6.8.3 Relative Lung weight

The external morphology of lungs showed normal architecture for vehicle control group (Gp. I) as depicted in **Fig. 68a**. However, tumor nodule was noticed in two out of five animals injected with B(a)P (Gp. II) as shown in **Fig. 68b**). The formation of tumor nodule was not observed for B(a)P injected animals administered with green tea (Gp. III), *O. gratissimum* (Gp. IV) and their combination (Gp. V) (**Fig. 68c-e**) **Table 30**.

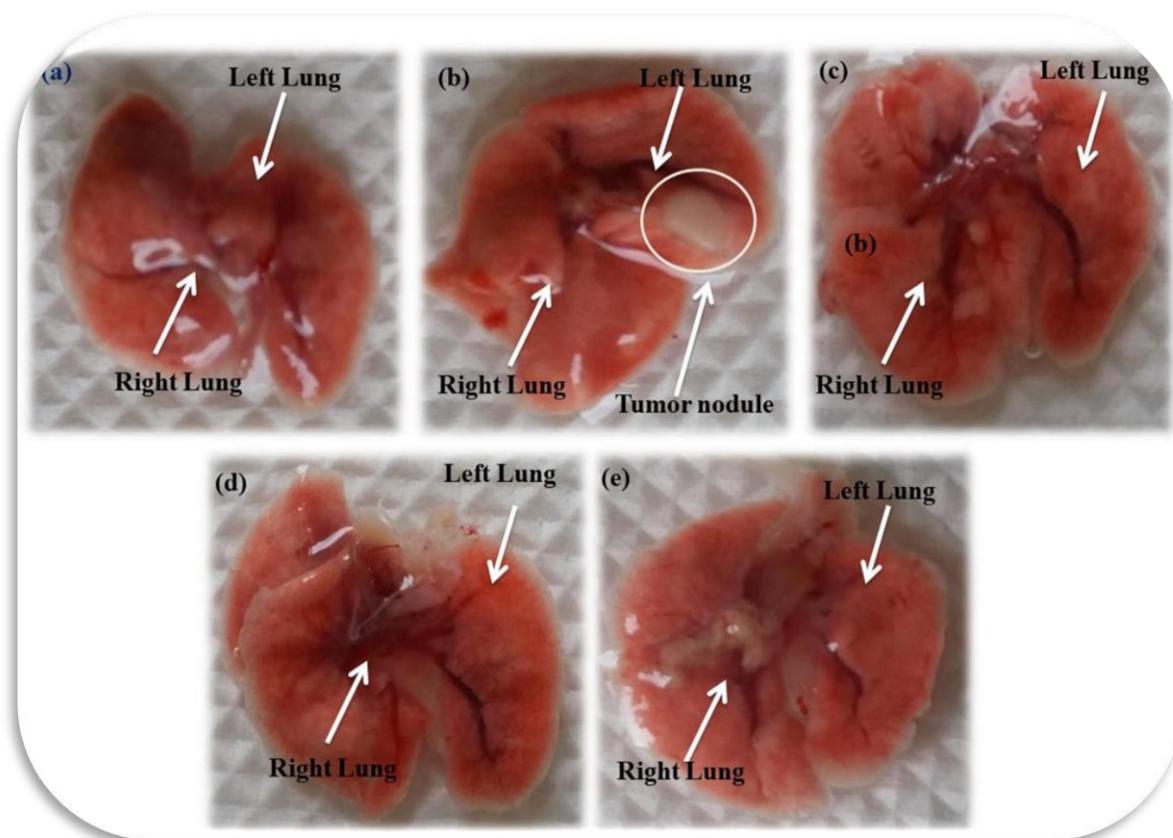


Fig. 71: Effect of GT, OG and their combination on external morphology of lungs against B[a]P- induced preneoplastic lung lesions: (a) Gp. I (Control), (b) Gp.II (Benzo(a)pyrene), (c) Gp.III (green tea), (d) Gp. IV (*O. gratissimum*) and (e) Gp. V (green tea and *O. gratissimum*).

6.8.4 Evaluation of lung histological changes

Haematoxylin and eosin stained lung sections were evaluated microscopically. The internal structure of lungs of mice showed presence of bronchiole, the terminal bronchiole, alveolar ducts, alveolar sac and alveoli. The bronchiolar epithelium was found to have simple cuboidal cells and copious blood vessels. The existence of ciliated cells covered with cilia and dome shaped clara cells lining the bronchiole of lung tissues were observed. A subdivision of bronchioles leading into alveolar ducts known as terminal bronchiole was observed. The alveoli and smooth muscles lead the formation of alveolar ducts. The alveoli give rise to a sphincter like structure called the alveolar sacs that are present in the lung tissue. It is the last section of the respiratory region. The alveoli arising from the nearby alveolar ducts and respiratory sacs are connected through pore in the alveolar septa. The structural and functional component of the respiratory region is called the alveolus is also

present in the lung tissue. The type I squamous (thin and flat in shape) and type II cells (cuboidal or round in shape) are also present there in the alveolar surface **Fig. 72a** . The lung lesions are classified as alveolar, bronchiolar, alveolar/bronchiolar hyperplasia and adenoma. Gp. I animals demonstrated normal architecture of lung sections showing single layer of bronchiolar and alveolar epithelium (**Fig. 72a-d**).

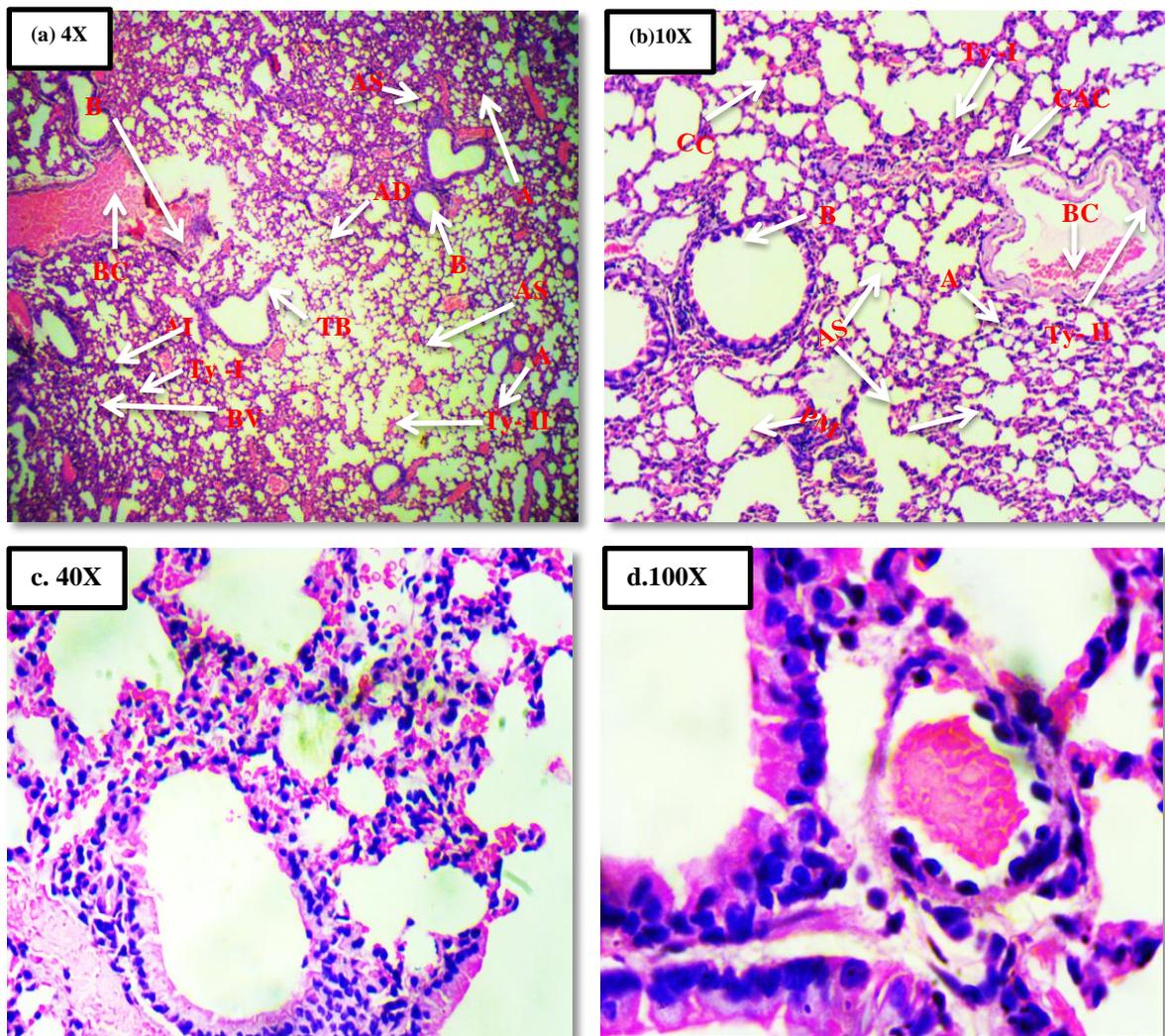
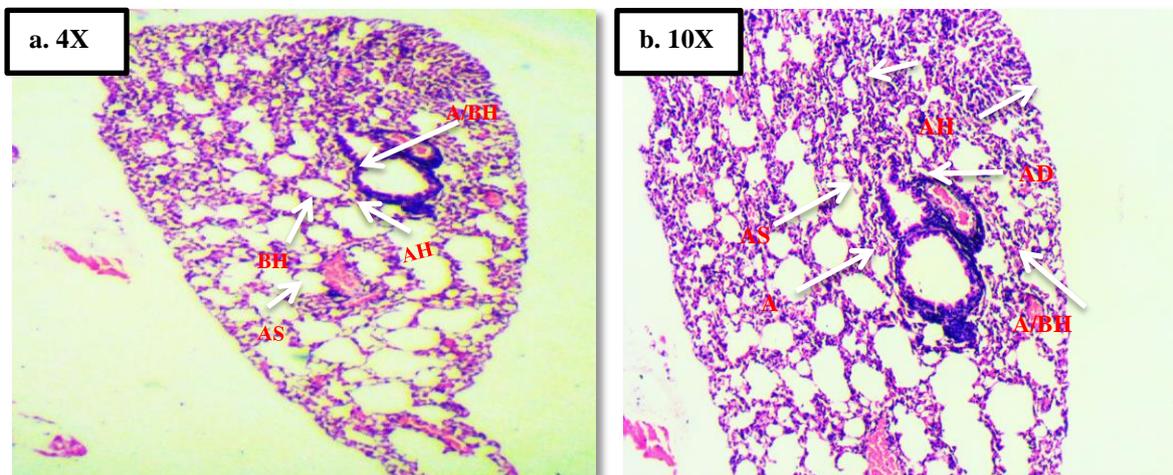


Fig. 72: Lung Histology of Gp. I animals showing normal architecture of lung tissue (hematoxylin and eosin staining) viewed under light microscope [a. 4x, b. 10x, c. 40x and d. 100x]. Bronchiole (B), Terminal bronchiole (TB), Alveolar ducts (AD), Alveolar sac (AS), Alveolus (A), Alveoli (AI), Blood capillaries (BC), Ciliated cells (CC), Pulmonary macrophages (PM), Clara cells (CAC), Type I (Ty-I) and Type II (Ty-II).

Gp. II [B(a)P] animals revealed alterations in the lung histo-architecture. The hyperplastic changes in bronchiolar and alveolar epithelium was observed at certain sites. In these regions single layer of bronchiolar and alveolar cells was replaced with multiple layers of cells (hyperplasia) **Fig. 73**. The alveolar and bronchiolar structure was found to be distorted and the bronchioles and alveolus was found to possess large and darkly stained nuclei. The epithelial lung lesions such as alveolar, bronchiolar, bronchiolar-alveolar hyperplasia and adenoma (two out of five) was detected in B(a)P injected (Gp. II) lung tissues. The hyperplastic zones were observed in all animals given B(a)P, but variation with the number of these zones were observed within the same group **Fig. 77** and **Table 31**. The lung alveolar/bronchiolar epithelial hyperplasia is observed over some regions of the lung sections, these structures are formed by involvement of terminal bronchiole, alveolar ducts and alveoli (centriacinar region) of the lung. The presence of large and foamy macrophages, and few inflammatory cells was also noticed. The distinct nodules of neoplastic epithelial tissue in the pulmonary parenchyma was observed in two out of five animals of this group. The neoplastic cells form and size are polygonal or round with the least amount of cytoplasm. It consists of uniform epithelial cell population and nuclei with coarsely clumped chromatin of irregular shape.



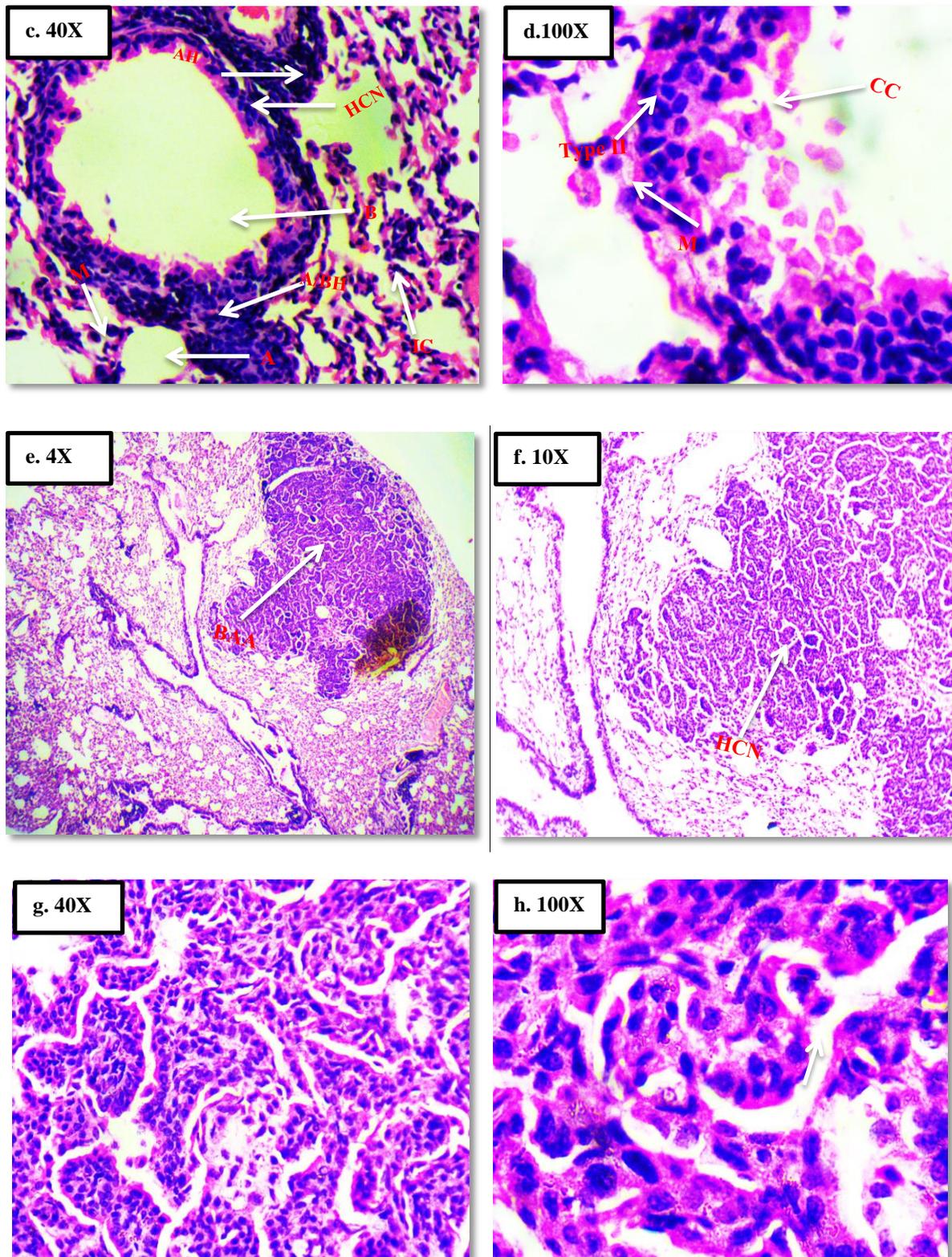


Fig. 73: Histopathological analysis of Gp. II B(a)P] showing the alveolar, bronchiolar, bronchioloalveolar hyperplasia and adenoma (hematoxylin and eosin staining) viewed under light microscope [a. 4X; b. 10X; c. 40X; d. 100X; e. 10X and f. 40X], bronchiole (B), Terminal bronchiole (TB), alveolar ducts (AD), Alveolar sac (AS), alveolus (A), alveoli (AI), ciliated cells (CC), macrophages (M), clara cells (CAC), bronchiolar

hyperplasia (BH), alveolar hyperplasia (AH), alveolar/bronchiolar hyperplasia (A/BH), hyperchromatic nuclei (HCN) and bronchioloalveolar adenoma (BAA).

The oral administration of green tea, *O. gratissimum* and their combination reduce the number of pulmonary lesions in the Gp. III, Gp. IV and Gp. V, respectively **Fig. 74**. The incidence of B(a)P induced alveolar, bronchiolar and alveolar/bronchiolar hyperplasia declined to 72.73%, 75.00% and 77.28% in green tea treated group (Gp. III) in comparison to only B(a)P injected group (Gp. II) animals ($p \leq 0.05$) as depicted in **Fig. 77** and **Table 31**. The infiltration of inflammatory cells was found to be less in the bronchiolar region as compared to group II animals.

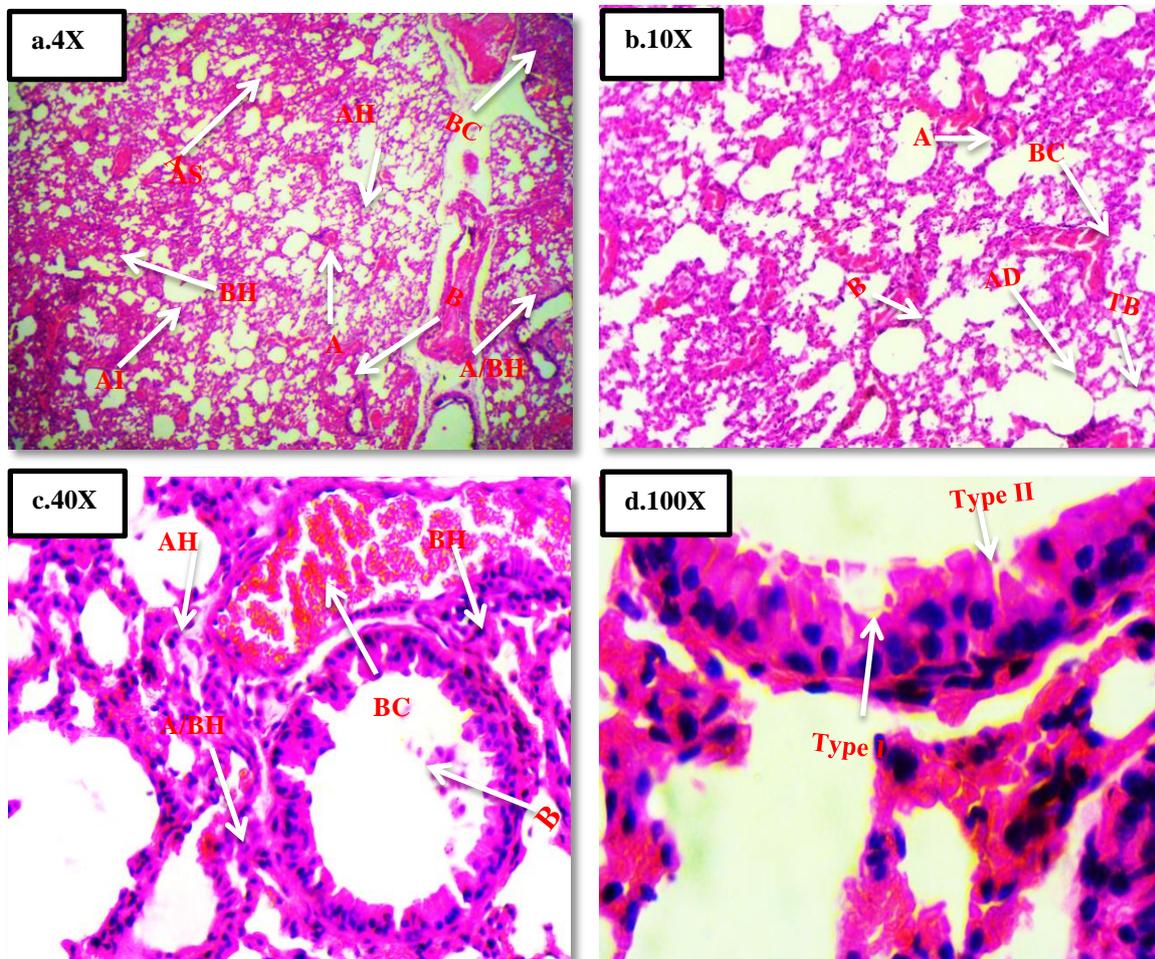


Fig. 74 Histopathological analysis of Gp. III (green tea) showing the reduction in alveolar, bronchiolar, bronchioloalveolar hyperplasia (hematoxylin and eosin staining) viewed under light microscope [a. 4X; b. 10X; c. 40X; d. 100X], Bronchiole (B), Terminal bronchiole (TB), alveolar ducts (AD), Alveolar sac (AS), alveolus (A), alveoli (AI), blood capillaries (BC), macrophages (M), bronchiolar hyperplasia (BH), alveolar hyperplasia (AH) and alveolar/bronchiolar hyperplasia (A/BH).

B(a)P induced hyperplastic regions for *O. gratissimum* administered animals (Gp. IV) was also found to be reduced, but the reduction was similar as compared to green tea treated mice for alveolar, bronchiolar and alveolar/bronchiolar hyperplastic regions as depicted in **Fig. 75** and **Table 31** The alveolar, bronchiolar and alveolar/bronchiolar hyperplasia was reduced to 54.55%, 75.00% and 59.09%, respectively as compared to Gp. II animals ($p \leq 0.05$) **Fig. 77**. Compared to group II mice, the hyperchromatic and unequal nuclei in the cells of the alveolar wall were reduced.

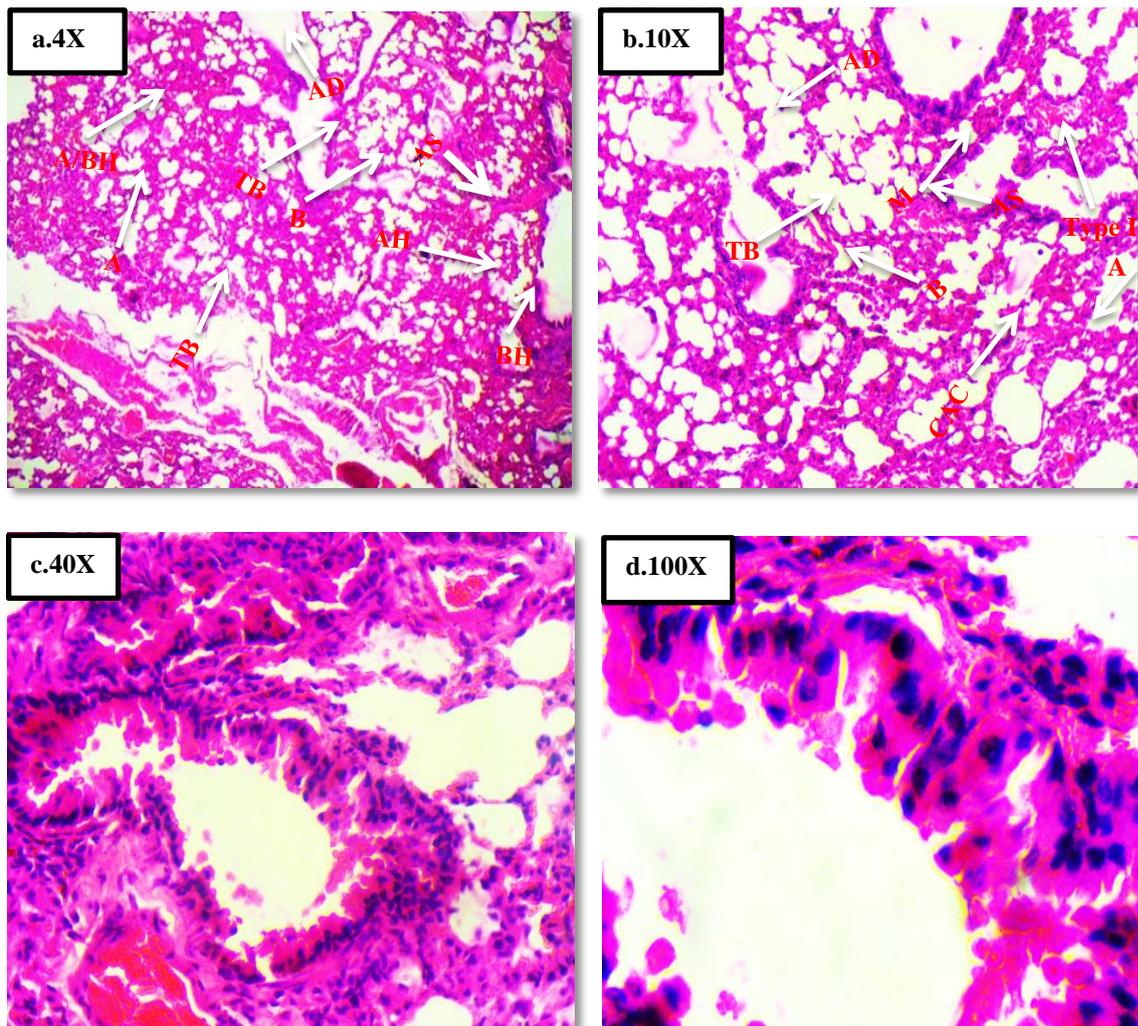


Fig. 75: Histopathological analysis of Gp. IV (*O. gratissimum*) showing the reduction in alveolar, bronchiolar, bronchioloalveolar hyperplasia (hematoxylin and eosin staining) viewed under light microscope [a, 4X; b, 10X; c, 40X; d, 100X], bronchiole (B), Terminal bronchiole (TB), alveolar ducts (AD), Alveolar sac (AS), alveolus (A), alveoli (AI), blood capillaries (BC), macrophages (M), clara cells (CAC), bronchiolar hyperplasia (BH), alveolar hyperplasia (AH) and alveolar/bronchiolar hyperplasia (A/BH).

The binary combination of green tea and *O. gratissimum* (Gp. V) animals showed decline in hyperplastic regions similar to Gp. III and Gp. IV as compared to Gp. II **Fig. 76**. The alveolar, bronchiolar and alveolar/bronchiolar hyperplasia declined to 68.10%, 75.00% and 72.73%. There was observed a significant reduction in the number of alveolar, bronchiolar and bronchiolar/alveolar hyperplastic zones of Gp. V animals compared to Gp. II ($p \leq 0.05$) was observed **Fig 77**. However, the decline in lung lesions (similar bronchiolar, alveolar and alveolar/bronchiolar hyperplastic zones) were noticed to be in Gp. III, Gp. IV and Gp. V animals ($p \geq 0.05$). Moreover, adenoma formation was not detected in these groups **Table 31**.

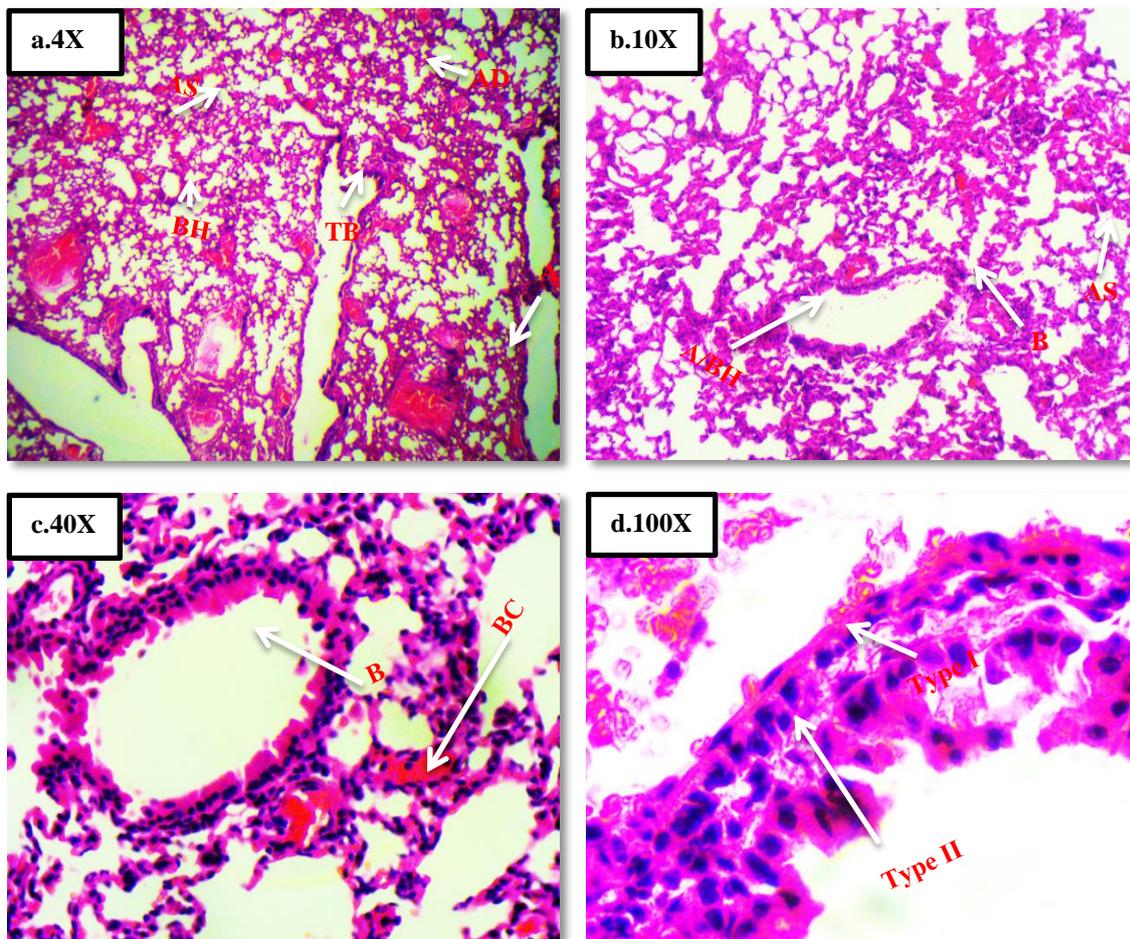


Fig. 76: Histopathological analysis of Gp. V (green tea and *O. gratissimum*) showing the reduction in alveolar, bronchiolar, bronchioloalveolar hyperplasia (hematoxylin and eosin staining) viewed under light microscope [a, 4X; b, 10X; c, 40X; d, 100X], bronchiole (B), Terminal bronchiole (TB), alveolar ducts (AD), Alveolar sac (AS), alveolus (A), alveoli (AI), blood capillaries (BC), bronchiolar hyperplasia (BH), alveolar hyperplasia (AH) and alveolar/bronchiolar hyperplasia (A/BH).

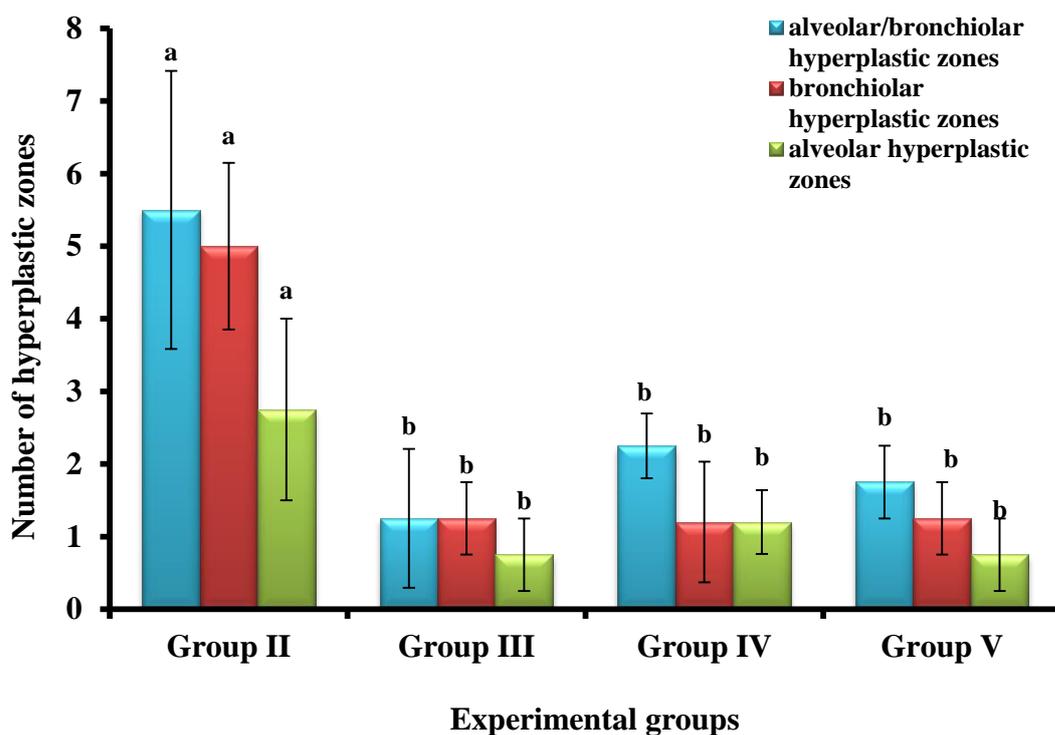


Fig. 77: The protective effect of GT, OG and their combination on number of hyperplastic zones during lung tumorigenesis induced by benzo(a)pyrene. Values are signified as mean \pm S.D. Dissimilar alphabets demonstrated a large difference among the zones of different experimental groups separately ($p \leq 0.05$).

Table 31: Incidence of Alveolar, Bronchiolar and Alveolar/bronchiolar hyperplastic regions in different B(a)P induced experimental groups.

| Groups | Alveolar hyperplastic regions | Bronchiolar hyperplastic regions | Alveolar/Bronchiolar hyperplastic regions | Total hyperplastic regions |
|-----------|-------------------------------|----------------------------------|---|------------------------------|
| Group I | - | - | - | - |
| Group II | 2.75 \pm 1.25 ^a | 5.00 \pm 1.15 ^a | 5.50 \pm 1.91 ^a | 4.41 \pm 0.80 ^a |
| Group III | 0.75 \pm 0.50 ^b | 1.25 \pm 0.50 ^b | 1.25 \pm 0.95 ^b | 1.08 \pm 0.25 ^b |
| Group IV | 1.25 \pm 0.44 ^b | 1.29 \pm 0.80 ^b | 2.25 \pm 0.44 ^b | 1.59 \pm 0.20 ^b |
| Group V | 0.75 \pm 0.50 ^b | 1.25 \pm 0.50 ^b | 1.75 \pm 0.20 ^b | 1.25 \pm 0.40 ^b |

Values are embodied as MEAN \pm S.D. The dissimilar alphabets indicated significant difference ($p \leq 0.05$) among the hyperplastic zones of different experimental groups separately. Gp. II [B(a)P], Gp.III (green tea), Gp. IV (*O. gratissimum*) and Gp. V (green tea and *O. gratissimum*).

CHAPTER 7
DISCUSSION

DISCUSSION

The findings revealed that various individual aqueous infusions and GT combinations may act differently against several radicals. This may be due to different reagents used in each method and stereochemical structure of these radicals. GT and herbal teas (OG, CC, CF and HR) illustrated applicable antioxidant capacity. Previous studies also reported a suitable antioxidant activity of green tea and other herbs (lavender, jasmine, sweet osmanthus, rose, rosemary, daisy, lemon balm, peppermint, lemongrass, oregano, sage, greek oregano and winter savory) (Tsai et al., 2007; Chrpova et al., 2010). Cell free models (DPPH, ABTS and NO) and *ex vivo* models (LPO and haemolysis) were used for antioxidant potential determination. Among the individual infusions, GT exhibited the lowest EC₅₀ (highest antioxidant potential) in all the assays performed (DPPH, ABTS, NO, LPO, and haemolysis) except ABTS, where GT revealed similar activity to that of HR and in case of anti-lipid-peroxidation assay, GT activity was similar to CF as demonstrated in **Fig. 17-21** and **Table 12**. DPPH is a free radical model that is commonly used to analyze antioxidant capacity of food products, phytochemicals, plant extracts, and beverages (Mathew and Abraham, 2006). ABTS is an unstable colored free radical and can be dissolved both in aqueous and organic phases. This feature of ABTS can make it capable to find the antioxidant activities of both hydrophobic and hydrophilic antioxidant of food extracts (Kim et al., 2002). Previous *in vitro* studies also reported strong DPPH radical activity of GT as compared to different herbs such as *Phyllanthus emblica*, *Vitis vinifera*, *Punica granatum*, *Cinnamomum cassia* and *Ginkgo biloba* (Jain et al., 2011). Green tea aqueous infusions suggested greater radical scavenging activity for DPPH and ABTS relative to oolong and black tea (Islam et al., 2018). Among 112 studied aqueous extracts of Chinese medicinal plants, GT demonstrated the sixth highest ABTS radical scavenging capacity (Cai et al., 2004). A study investigated 18 tea infusions among which green tea demonstrated maximum ABTS and FRAP radical scavenging potential and licorice herbal tea exerts the minimum activity (Deetae et al., 2012). NO is a free radical formed from sodium nitroprusside that forms nitrite when reacting with oxygen. By competing with oxygen, the extracts scavenge nitrite formation and decrease the production of NO (Marcocci et al., 1994). In the control of different physiological procedures, nitric oxide plays an important role and excessive generation of this radical is toxic to cells and tissues that can cause different disorders (Choudhari et al., 2013). Aqueous extract of GT demonstrated highest nitric oxide scavenging activity followed by rosemary, pleasant osmanthus, jasmine, lemongrass, rose, lavender, and daisy extracts (Tsai et al.,

2007). ROS induce lipid peroxidation, damage to cellular membranes, lipoproteins and other molecules containing lipids in oxidative stress conditions. The PUFA (polyunsaturated fatty acids) are more susceptible to free radical damage and lead the formation of lipid peroxides (Nawrot et al., 2008). Free radicals are initiators and terminators of lipid peroxidation processes. When activated, the reaction proceeds auto-catalytically; it has a progressive path and the end result is structural and functional substrate changes (Cuypers et al., 2010). It was reported that, among the aqueous infusions of black tea, white and green tea, the green tea revealed the second highest anti-lipid peroxidation activity by using chick liver model (Kaur et al., 2019). Earlier investigations on green, white and black tea showed that these infusions have the capacity of inhibiting lipid peroxidation (Alarcon et al., 2008; Islam, 2011; Kumar et al., 2012). Human erythrocytes are commonly used to research the effect of plant extracts on the H₂O₂-induced hemolysis as a model system (Farooq and Sehgal, 2017). Oxidative damage to the red blood cell membrane, used in current report is a simple and suitable model for illuminating the redox mechanism. GT leaf extract was found to be more effective in ameliorating phenylhydrazine-mediated lipid peroxidation of erythrocytes as compared to ascorbic acid (Biswas et al., 2005)

Plants are having high content of phenolic and flavonoids that are responsible for their potential antioxidant capacity contributing to their various defensive and disease fighting properties (Kahkonen, et al., 1999; Manssouri et al., 2020). The antioxidant ability of phenolic and flavonoid compounds is mainly due to their redox properties that make them act as reduction agents, donors of hydrogen and quenchers of singlet oxygen. They may also have a potential for chelating metals (Mradu et al., 2012). Green tea demonstrated higher phenolic and flavonoid content among all the individual infusions (OG, CC, CF and HR) as depicted in **Fig. 28** and **29** and **Table 13**. Previous study reported that green tea, accompanied by oolong and black tea, recorded the highest total phenolic and flavonoid content (Islam et al., 2018). Another study reported higher TPC/TFC in green tea and low- caffeine green tea followed by white, black CTC and orthodox tea except for TFC in which white tea displayed lower value than black orthodox tea (Carloni et al., 2013). The individual infusions showed a high correlation between the antioxidant activity and TPC/TFC except anti-lipid peroxidation activity in which very weak correlation was observed as displayed in **Table 14**. A positive association between the phenolic content of tea and the antioxidant activity of green, white and black tea was also observed, with an increase in TPC resulting in an increased radical DPPH scavenging potential of tea infusions (Pereira et al., 2014). Green tea's antioxidant

activity was found to be strongly associated with its polyphenolic material (Deetae et al., 2012). The variations in the antioxidant activity of green tea and other teas may be due to differences in various factors like form, conditions of cultivation, agricultural practices, cultivar, leaf age, grade, topographical conditions, storage and processing and quantity of polyphenols found in various teas (Damiani et al., 2014; Farooq and Sehgal, 2018).

The mixing of plant extracts may change the biological effect of that particular extract. According to the interaction of the components, it may be either higher or lower than the summative effects of each component alone. It is possible to identify phytochemical interactions as synergy, antagonism or additive (Phan et al., 2018). The mixture of compounds functions in such a way that if one compound fails to perform its function and another is available to fulfill its action (Boik, 2001). Traditionally, used medicinal plants consumed as aqueous infusions or herbal teas or tisanes with proven record of antioxidant potential were combined with GT. It was observed that combining different medicinal plants showed higher antioxidant capacity than the individual plant (Yang et al., 2009). The strength of combination of herbs is not only increased by increase in concentration of individual herbs but also by the interaction between various herbs with different pharmacological properties (Jia et al., 2004). Therefore, examining the interaction types different methods must be carried out (Skroza et al., 2015). In the current investigation, various techniques such as isobolograms, combination index and polygonogram were employed to evaluate the type of interaction between the binary mixtures of GT. It was found that GT binary infusions showed synergistic to additive interactions and the type of interaction may vary in different antioxidant assays as given in **Fig. 22-26** and **Table 12**. This might be due to the interaction of different type of phytochemicals that are present in binary combinations. Earlier studies also reported variation in antioxidant interactions of binary mixtures of various plants extracts in different antioxidant tests (Gawlik-Dziki, 2012; Liu et al., 2016). Interestingly, GT+OG combination showed synergistic interactions (synergism to slight synergism) and lowest EC₅₀ in all the antioxidant assays performed as shown in **Table 12**. While other GT combinations demonstrated contrasting interaction types such as GT+CC (moderate synergism to nearly additive), GT+CF (slight synergism to nearly additive), and GT+HR (moderate synergism to nearly additive) depicted in **Table 12**. The interaction may occur in the phytochemicals of the whole extract of a single herb or between different herbs in a combination (Cooper et al., 2005). Some studies showed synergistic antioxidant interaction of green tea or green tea polyphenols with medicinal plants such as *Osmanthus fragrans* (flowers) or *Potentilla*

fruticosa (leaves) (Dai et al., 2008; Mao et al., 2017), while other reports revealed synergistic to antagonistic interactions between GT and other plants, and its own phytoconstituents (Liu et al., 2016; Nedamani et al., 2015). The antioxidant interactions between equimolar proportion of pairs of phenolic compounds ranged from synergistic (rosmarinic acid-caffeic acid/quercetin), additive (rosmarinic acid-(+)-catechin), or antagonistic (caffeic acid-quercetin/(+)-catechin), and these mixture effects have been partially assigned to mechanisms of regeneration between antioxidants, depending on the phytochemical structure and the possible formation of stable complexes between molecules (Peyrat-Maillard et al., 2003). The synergistic interaction between the phytoconstituents may develop due to multiple mechanisms such as regeneration, spatial distribution, sacrificial oxidation, reciprocal defense, and metal chelation of the more effective antioxidant by the less effective antioxidant (Fuhrman et al., 2000; Neunert et al., 2015; Yan, 2001). The antioxidant regeneration is cited as the most common method for explaining the synergistic interactions between phytochemicals or polyphenolic mixture (Fuhrman et al., 2000). The synergistic effect can be observed when two or three herbs are mixed together that may form a new compound with higher antioxidant potential (Yan, 2001). In agreement to our study, binary mixtures of green tea extract with green tea catechins (epicatechin, gallic catechin, catechin and epigallocatechin) revealed additive effect (Colon and Nerin, 2016). The additive effect is due to no interactions between the various phytochemicals or antioxidants that cause these substances in the mixture to behave the same as the individual and unknown mutual interactions between the antioxidants (Olszowy-Tomczyk, 2020).

However, TPC/TFC was found to decrease notably in GT combinations when compared to their average theoretical value except in GT+CC and GT+CF where a slight increase was found in the experimental value of TFC as depicted in **Table 13**. Most of the previous findings for individual or combination of plant extracts suggested that increase in phenolic content is associated with increase in radical scavenging potential (Yang et al., 2009). It is thought provoking that even there is decline in phenolic content in all investigated GT combinations, but the antioxidant interactions ranged from synergistic to additive. This may be due to chemical interaction of different components with each other that can alter structural characteristic of polyphenols and may also lead to polymerization of monomer to dimer (Cho et al., 2006). These changes may possibly affect the estimation of TPC by Folin–Ciocalteu method. Few studies reported that binary combination of certain pairs of herbs demonstrated a decrease in phenolic and flavonoid content but increase in antioxidant activity

as compared to their individual samples, such herb pairs are *Astragalus membranaceus-Angelica sinensis*, *Paeonia lactiflora-Glycyrrhiza uralensis*, *Paeonia lactiflora-Angelica sinensis*, *Astragalus membranaceus-Glycyrrhiza uralensis*, Tomato-Garlic, and Tomato-Lettuce (Gawlik-Dziki, 2012; Ivanova et al., 2005; Liu et al., 2016).

The ATR-FTIR spectra of evaluated infusions (individuals infusions, green tea combinations, and GT and OG combination at different ratios) containing multiple bands, each band may represent an overall overlap of some characteristic absorption peaks. The spectral pattern of all single or binary infusions was similar, but differences are observed in intensities, width of peaks and also minor shift in wavenumbers. The differences in absorption intensities may be due to variation in number of functional groups corresponding to that particular range of wave number. In current study, all the single and binary infusions demonstrated a major peak in the region between 3000-3600 cm^{-1} , other peaks in the range of 2800-3000, 1400-1700, 1250-1400, 1100-1250, 950-1200 cm^{-1} and 700-900, respectively. In agreement to our study previous reports also revealed broad peaks of green tea, *O. gratissimum*, *C. citratus*, *C. flexuosus* and *H. rosa sinensis* samples in the region between 3000-3600 cm^{-1} assigned OH stretching modes of alcohols/phenols/carboxylic acids and NH stretching of amines (Loo et al., 2012; Senthilkumar and Sivakumar, 2014; Dubey et al., 2017; Irshad et al., 2018; Petchsoongsakul and Pechyen, 2012; Sathiyamoorthi and Sankaranarayanan, 2017). This suggests that broad natured band may be originated from the overlapping of OH and NH stretching mode of vibration of alcohol/water present in the plant extracts. Similar to our study, bands in the region between 2800-3000 cm^{-1} were observed in GT, OG, CC, CF and HR samples demonstrated the CH stretch of alkanes and OH stretch of carboxylic acids (Murugan et al., 2015; Senthilkumar et al., 2017). The samples of GT, OG, CC, CF and HR revealed peaks in the region between 1400-1700 cm^{-1} centered at different wavenumbers due to C=C and C=O stretch of aromatic compounds and carboxylic acids (Jain and Mehata, 2017; Murugan et al., 2015; Mak et al., 2013; Kamaruddin et al., 2017; Senthilkumar and Sivakumar, 2014; Gupta and Rastogi, 2008). There also occurs a set of small absorption peaks in the region 1600- 2000 cm^{-1} IR spectra of all samples known as overtones (the multiples of the fundamental absorption frequency). The other peaks between the range of 1250-1400 cm^{-1} were also observed in GT, OG, CC and HR extracts, these can be due to CH stretching of alkanes, CN stretch aliphatic amines and CO stretch alcohols/ether/carboxylic acids/ether (Mak et al., 2013; Thangam et al., 2014; Kadhim et al., 2016; Senthilkumar et al., 2017). Previous studies demonstrated the peaks in the region between 1100-1250 cm^{-1} for

GT, OG and HR samples assigned CO stretching of alcohols/esters and carboxylic acids (Mak et al., 2013; Kadhim et al., 2016; Senthilkumar et al., 2017). GT, OG and CC samples previously showed peaks in the region between 950-1100 cm^{-1} assigned CO stretching of alcohols/carboxylic acids/ethers (Udunwa et al., 2017; Senthilkumar et al., 2017). One of the report demonstrated that peak centered at 1041.60 cm^{-1} for GT with OH stretching of trihydroxybenzoate of epicatechingallate C-O-C group which associates the chromane ring and trihydroxy benzoate ring (Robb et al., 2002; Gopal et al., 2016). The absorption bands in the range of 700-900 cm^{-1} were also observed earlier in GT, CF and OG samples associated to C-H bending of aromatic compounds (Kadhim et al., 2016; Gopal et al., 2016). For individual samples, the maximum peak intensity was observed for GT and minimum in case of CC in 3000-3600 cm^{-1} region. The intensity of peaks was found to be similar in GT and OG spectra, and CF and HR spectra in the range 3000-3600 cm^{-1} . There were observed some peaks in the individual samples that are having same wavenumbers such as CF and CC (1035.82 cm^{-1}), OG and HR (1199.76 cm^{-1}), CF and HR (923.93 cm^{-1}), and GT and CF (810.13 cm^{-1}), respectively. This may be due to some similar type of phytochemicals present in these extracts. Interestingly, GT combinations demonstrated similar spectra, as that of individual infusions except few additional peaks in the region 3600–4000 cm^{-1} assigned to free OH of alcohol (Siripatrawan and Harte, 2010). Previous study also demonstrated similar spectral profiles of green tea samples from five different brands (Li et al., 2018). The GT+OG combination demonstrated maximum peak intensity followed by GT+HR, GT+CF and lower in GT+CC combination as shown in **Fig. 40**. The major contributor to absorbance in this region (3000–3600 cm^{-1}) is phenolic hydroxyl groups, and was found that higher the number of phenolic OH groups, more the radical scavenging potential (Lopez-Velez et al., 2002; Mira et al., 2002). Similar absorption bands in FTIR spectra were identified in all the analyzed ratios of binary mixture, but their intensities and broadness were slight different. The spectra of our green tea sample matches the characteristic band of EGCG as it is the major component of green tea (Ponnuraj et al., 2015). It was noticed that spectra of various ratios of GT and OG combination were similar to that of green tea. The broadness and the intensity of this peak (3000-3600 cm^{-1}) varied in various ratios. The samples of various ratios displayed similar type of FTIR characteristic bands as that of earlier reports for these plant extracts (Ramamurthi and Kannan, 2007), however some reports showed different spectra (Bobby et al., 2012; Pednekar and Raman, 2013). GT showed more intense and broad peak followed by 3:1, OG, 1:1, 2:1, 1:2 and 1:3 as illustrated in **Fig 62**. It may be because of the high phenolic content present in the GT infusion. However, 1:1 showed more intense peak than 2:1 that

may be due to different proportion of various phytochemicals that may alter the phenolic content of the extract. For the occurrence of mixing potential between GT and OG extract, the shifting in the FTIR bands and the decrease in intensity are obvious. This may be due to different proportions of GT and OG combination.

For all samples individual or binary mixtures or ratios, the intensity of the absorption peak (3250–3290 cm^{-1}) and TPC or antioxidant activity, were considered for correlation analysis. For individual samples, very high correlation was observed between the peak intensity and TPC or antioxidant activity. A negligible correlation for LPO, moderate for ABTS and haemolysis, high in DPPH and very high correlation in NO assay was observed as given in **Table 16**. However, for binary mixtures, correlation between antioxidant activity and peak intensity was noticed to be low in ABTS, moderate in LPO and NO, high in DPPH and very high in haemolysis assay, whereas negligible correlation with TPC was observed **Table 18**. This may be due to interaction of different phytochemicals present in these combinations with the Folin-ciocalteu reagent that might change the structural characteristic of phenolic components that may alter their estimation. For ratio study a high correlation between TPC and the intensity of peak, but negligible correlation between EC_{50} and intensity of the peak in all performed parameters as illustrated in **Table 25**. A study demonstrated an excellent correlation between the FTIR and colorimetric data (DPPH, FRAP, TPC and TF) of green tea samples (Senthilkumar et al., 2017). FTIR provides us the opportunity to get the preliminary understanding of polyphenolic structure of different samples. However, it is notable to discuss here that results obtained from FTIR analysis alone are not ample to prove the existence of compounds in different plant samples, especially when it comes to binary or ternary mixtures. There is a need to explore it by using some other high standard techniques such as advanced chemometrics, the NMR (nuclear magnetic resonance spectroscopy) and HPLC (high performance liquid chromatography).

Besides FTIR, UV- VIS investigation was also done. Among the individual samples only GT demonstrated an intense peak in the region 250-300nm as depicted in **Fig. 41**. This peak may be due to presence of catechins and caffeine present in green tea infusion that may be absent in other infusions. However, in all GT combinations (GT+OG, GT+CC, GT+CF and GT+HR), an intense peak in the region 250-300nm was observed, that was found very less in individual samples as displayed in **Fig. 42**. This may be due to mixing of GT phytochemicals in the binary combination that displayed this peak in all the binary mixtures. As GT was mixed with different herbs, the intensity of the absorbance increased, indicating the presence

of catechins in binary mixture. The UV-VIS analysis of GT+OG combination at various ratios displayed intensity of peak in the order GT, 3:1, 2:1, 1:1, 1:2,1:3 and OG respectively. OG revealed the least peak in the region 250-300nm. As earlier study reported that green tea catechins such as EGCG showed absorbance in the range (248-361nm) with λ_{\max} at 276.6nm, EGC (254-378nm) with λ_{\max} at 269.6nm, ECG (246-363nm) with λ_{\max} 276.8nm and EC (252-328nm) with λ_{\max} 278.9nm (Atomssa and Gholap, 2015). The maximum peak intensity for caffeine was found in the region of 243- 302 nm at room temperature with λ_{\max} at 272nm (Belay and Gholap, 2009). However, in case of the green tea there was found that 20K-P particles having higher flavonoid content demonstrated an increase in EGCG intensity compared to the 5K-P and 10K-P (Gopal et al., 2016). There was found a high correlation between absorbance (200-300nm) and TPC of the various samples. A very high correlation with absorbance (200-300nm) and EC₅₀ of the individual samples in haemolysis assay, high in NO, DPPH and ABTS assay, whereas negligible in LPO as given in **Table 19**. A moderate correlation was found between TPC and absorbance of different GT combinations, but high correlation was there between EC₅₀ and absorbance of different samples in haemolysis assay, moderate in DPPH and negligible in ABTS, NO and LPO assays as shown in **Table 20**. Which might be due to major catechin content in the GT that interact with different phytochemicals of other samples and alter the absorption of binary mixtures in that region. GT and OG combination at various ratios displayed low correlation between TPC and absorbance in the region 250-300nm, high correlation in DPPH, ABTS and NO assays while moderate in LPO and haemolysis assay given in **Table 26**. One of the reasons may be interaction of different phytochemicals at various ratios with the Folin-Ciocalteu reagent that may alter the properties of polyphenols.

Previous studies also pointed out that the ratio of the herbs would influence the antioxidant properties (Liu et al., 2016; Muhammad et al., 2017). It is important to investigate the modulation of antioxidant properties depending on the proportions of herbs in a mixture that can be used to design functional foods and pharmaceutical products at different concentrations (Enko and Gliszczynska-Swiglo, 2015). The proportion of extracts significantly affected a binary combination's antioxidant ability and the form of interaction between their bioactive components (Enko and Gliszczynska-Swiglo, 2015; Jiang et al., 2015; Muhammad et al., 2017). In current research, binary combination of whole leaf aqueous extracts of green tea and *O. gratissimum* at different ratios was also evaluated, simulating the household method of tea preparation. It was analyzed that green tea and *O.*

gratissimum (GT+OG) at different ratios influenced the radical scavenging ability in various parameters. The proportion of the individual extracts alters the antioxidant ability of the binary combination in different *in vitro* models like DPPH, ABTS, FRAP (ferric reducing antioxidant power) and Trolox equivalent antioxidant capacity (Enko and Gliszczynska-Swiglo, 2015; Jiang et al., 2015; Liu et al., 2016; Muhammad et al., 2017). Interestingly, increasing proportion of green tea (active extract) in a binary mixture leads to an increase/decrease or almost no alteration in antioxidant capacity in different assays as given in **Table 21**. Green tea-ascorbic acid pair, black tea-ascorbic acid pair, and cocoa-cinnamon pair demonstrated changes in antioxidant potential with an increase in the active component, but no alteration was noted at certain fractions (Enko and Gliszczynska-Swiglo, 2015; Muhammad et al., 2017). It was also observed that in PFE and GTP combination, with enhancement in the GTP (active extract) from 7:1 to 1:7, an increase in radical scavenging activity was detected, but decrease or no variation was also noticed at certain ratios (Liu et al., 2016; Muhammad et al., 2017). These investigations testified that an enhancement in the proportion of active extract will not always increase the activity. The single infusion demonstrating higher activity as compared to other individual extracts may illustrate higher/lower/similar activity as that of the most effective ratio of mixed extract (Enko and Gliszczynska-Swiglo, 2015; Muhammad et al., 2017). Green tea displayed either lower (NO, LPO and haemolysis assays) or similar potential (DPPH and ABTS assays) in comparison to GT+OG (1:1) as displayed in **Table 21**. The interaction between green tea and *O. gratissimum* at different ratios ranged from moderate antagonism to strong synergism. *Prunus fruticosa* and green tea polyphenols combination demonstrated mostly a synergistic interaction, but the additive effect was also observed in a few cases. However, for cocoa and cinnamon combination, all types of interactions were detected in DPPH and FRAP assays (Liu et al., 2016; Muhammad et al., 2017). These synergistic interactions may originate due to various mechanisms such as regeneration, spatial distribution, sacrificial oxidation, mutual protection and metal chelation (Fuhrman et al., 2000; Becker et al., 2004; Becker et al., 2007; Dai et al., 2008; Neunert et al., 2015). Antagonism was detected only in the DPPH assay at 1:2 as shown in **Table 21**. This interaction can be due to the regeneration by a more efficient compound of the less active antioxidant, rivalry between the development of antioxidant radical adducts and the regeneration of the antioxidant, and alteration of one antioxidant's microenvironment by another (Mortensen and Skibsted, 1997; Peyrat-Maillard et al., 2003). Green tea and *Ocimum gratissimum* (1:1) manifested the highest activities for NO and anti-haemolysis assays, and was comparable with 2:1 and 3:1 in DPPH, ABTS and LPO tests. The

extent of synergism was maximum at 1:1 in all antioxidant models except LPO where similar synergistic interaction was observed as given in **Table 21**. The antioxidant potentials and antioxidant interactions depend correspondingly on the nature of different radicals, structural characteristics of the paired compounds, the ratio of the bioactive mixtures and the reaction mechanism of tests (DPPH, ABTS, NO, LPO and haemolysis) (Jiang et al., 2015; Muzolf-Panek et al., 2012; Hidalgo et al., 2010).

The therapeutic value of various herbs and medicinal plants has been due to the existence of phenolic compounds (Djeridane et al., 2006). A positive correlation between TPC/TFC and the antioxidant ability of green and white tea extracts has been established (Rusak et al., 2008). It was expected that increasing the proportion of an infusion with high phenolic content in a binary mixture would enhance the TPC of the combination. In our study, it was noticed that by increasing the proportion of green tea, no change was noticed in TPC initially, after that an increase and then decrease in TPC was denoted as displayed in **Table 22**. The scientific data about the effect of the proportion of individual extracts on the phenolic content of the binary mixture is limited. A previous study reported that increasing the fraction of GTP, which has a higher contribution to phenolic content as compared to PFE in a binary combination leads to an increase in phenolic content from 1:7 to 7:1 (Liu et al., 2016). In existing study, the TPC was significantly lower than the theoretical value at different ratios for the GT+OG combination as given in **Table 22**. Earlier it was demonstrated that in a binary combination of PFE and GTP, the observed and expected value showed similar phenolic content, but at certain ratios, a significant difference was detected (Liu et al., 2016). The flavonoid content was similar for most of the ratios with a slight variation in certain fractions as depicted in **Table 22**. The individual infusions (green tea and *O. gratissimum*) showed strong correlation between antioxidant capacity and TPC/TFC, whereas weak to strong correlation was observed between antioxidant capacity and TPC, and almost no correlation was detected in case of TFC for GT+OG combinations illustrated in **Table 23**. This may be due to various mechanisms involved during the interaction of different components of individual infusions that may alter the structural characteristic of polyphenols and may also lead the polymerization of monomer to dimer (Okuda, 1999; Cho et al., 2006). These changes may possibly affect the estimation of TPC with Folin-Ciocalteu reagent. Green tea and *O. gratissimum* combinations displayed significant antioxidant potential, and 1:1 is the best proportion, showing the highest radical quenching ability and strongest synergism in both cell free and *ex vivo* models. Therefore, this study provides a scientific

basis for combining aqueous infusions at a particular ratio to attain maximum antioxidant potential.

The process of angiogenesis is characterized as the creation of new blood vessels from once pre-existing (Folkman, 2006; Rashidi et al., 2017). In our body the angiogenesis occur all the time via a series of biochemical steps commonly called “cascade” through which cells are made and secrete the substances that increases the growth of blood capillaries. When this process is complete some molecules called “factors” turn off the angiogenesis process. It plays a vigorous role in the pathogenesis of many diseases like psoriasis, rheumatoid arthritis, diabetic retinopathy, and cancer (Paleolog, 2002; Heidenreich et al., 2009; Chung and Ferrara, 2011). Cancerous cells make use of this normal process for other purposes that are to imbalance the angiogenesis activators that disallow the inhibitors and give the nearby tumor cells a ready contact to blood supply (Maiti et al., 2003). This process is regulated by various aspects like VEGF (vascular endothelial growth factor), bFGF (basic growth factor of fibroblasts) and PDGF (growth factor of platelets) (Folkman, 2003). Without angiogenesis, tumor cells cannot develop more than 1-2 mm³ in size and will die from hypoxia (Folkman, 1990), thus the key goal of cancer therapy is to block the development of new vessels (Carmeliet, 2005). Thus tumor angiogenesis is considered a promising challenge as therapeutic target for cancer therapy (Baluk et al., 2005; Ben-Mabrouk et al., 2016). During the last decade, a number of plant based compounds were investigated to have the anti-angiogenic capacity through various molecular pathways that have significant effect in the body (Lu et al., 2016). The chorioallantoic membrane (CAM) model is a delicate, easily possible and inexpensive *in vivo* test that is usually utilized to explore the angiogenic and anti-angiogenic property of various samples or compounds (Ribatti et al., 1996; Ribatti et al., 2000). CAM model was employed in present study to discover the modulation of angiogenesis by various aqueous infusions (GT, OG, CC, CF, HR, GT+OG, GT+CC, GT+CF and GT+HR, respectively). The results revealed that all infusions showed anti-angiogenic activity but GT and GT+OG samples revealed similar ($p \geq 0.05$) but higher reduction in vessel length compared to other infusions depicted in **Fig. 64, 65** and **Table 27**. The regular treatment of green tea and selenium rich green tea demonstrated less blood vessel formation as compared to control, showed the anti-angiogenic effects of green tea by using CAM model (Molan et al., 2019). The administration of GT decreases the tumor vessel density and xenograft size, as all tumors are dependent on angiogenesis (Sartippour et al., 200; Maiti et al., 2003). The EGCG administration also decreases the angiogenesis in the CAM model and

also significantly blocked the VEGF expression by suppressing the various pathways such as HIF-1 α and NF- κ B, therefore reducing the proliferation, migration, angiogenesis and the growth of breast tumor (Swiercz et al., 1999; Jung et al., 2001). *Ocimum gratissimum* extract reduced the cell number per microscope field and blood vessels restricted to the periphery of the martrigel plug (Nangia-Makker et al., 2007). The main component (citral) of *Cymbopogon citratus* demonstrated an anti-angiogenic activity by using the CAM model (Habib et al., 2020).

The balance between the therapeutic potential and toxic side effects of different infusions is important when evaluating its usefulness as a chemopreventive agent. For toxicity screening different methods are used in its evaluation. MTT and SRB tests are mostly used for cytotoxic screening of different materials (Vajrabhaya and Korsuwannawong, 2018). In 1990, the sulforhodamine B assay was developed and is one of the highly used methods to determine the *in vitro* cytotoxicity screening of different samples (Skehan et al., 1990). The SRB assay is more sensitive cytotoxicity method than the MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay and the relationship between cell number and absorbance is linear, even with high cell numbers (Skehan et al., 1990; Keepers et al., 1991). Furthermore, and contrary to the MTT test, the SRB test does not depend on metabolic reaction and results are independent of a variety of metabolic conditions. Taking into account, the *in vitro* cytotoxicity of the GT, OG and GT+OG samples against lung cancer cell line A549 by sulforhodamine B assay were evaluated. Lung cancer cell line was selected because the above mentioned infusions had to be further examined for *in vivo* lung cancer model. The results demonstrated that all infusions (GT, OG and GT+OG) revealed dose-independent reduction in viability of A549 cells at 48h. GT and GT+OG displayed comparable cytotoxic ability in a dose independent manner. These infusions illustrated greater inhibition of cell growth as compared to OG ($p \leq 0.05$) at low concentration whereas no difference was noticed at higher doses ($p \geq 0.05$) as depicted in **Fig. 66**. It was found that both aqueous and ethanolic extracts of GT showed the inhibition in the viability of A549 lung cancer cells (Li et al., 2008). Green tea extract and EGCG demonstrated similar reduction in cell growth of two lung cancer cell lines (PC-9 and PC-14) (Komori et al., 1993). At lower concentration of EGCG (30 μ M) showed no difference in the cell viability of different cells, but viability of cells was found to be much lower at higher concentration (100 μ M) (Yang et al., 1998). One of the study reported that the IC₅₀ values obtained from cytotoxicity data demonstrated clearly that black and green tea extracts are equally effective (Kundu et al.,

2005). However in another report the cytotoxic effect of green tea extract was found to be more noticeable in cancerous cells than the normal cells (Weisburg et al., 2004). A dose dependent reduction in the breast cancer cell viability was observed by the treatment of *O. gratissimum* and *O. basilicum* extracts, but *O. gratissimum* was observed to be more active at concentration 600µg/ml than *O. basilicum* (Torres et al., 2018). It was also reported that *O. gratissimum* extract reduced the A549 lung cancer cell viability 8%, 10%, 13%, 30% and 73%, respectively at 100, 200, 300, 500 and 800µg/ml concentration (Chen et al., 2010). The synergistic cytotoxicity effect was observed between red beetroot extract with doxorubicin against breast, pancreatic and prostate cancer cell lines (Kapadia et al., 2013). Morphological appearance is not that much clear in OG treated cells this might be due to consistency and composition of the *O. gratissimum* sample **Fig.67**.

In recent years huge struggles were made to improve the natural or synthetic chemopreventive agents that can slow or reverse the carcinogenesis process (Yan et al., 2005; Mohan and Koul, 2018). In current study, green tea and *O. gratissimum* combination were selected for *in vivo* experiment as this combination demonstrated the higher antioxidant potential and maximum synergistic effect at an equal ratio (1:1). Thus the aqueous infusions of green tea, *O. gratissimum* and their combination against benzo(a)pyrene induced lung histological changes in different experimental groups were evaluated.

The observations of present report illustrated no difference in water consumption and food intake in all experimental groups ($p \geq 0.05$) **Table 29**. A recent study reported, no difference in water consumption for B(a)P administration animals as compared to control group animals (Mohan and Koul, 2018). In agreement to our study no variation was detected in body and relative lung weight of different animal groups (Conaway et al., 2005; Manna et al., 2009; Yeo et al., 2017; Onami et al., 2017). As there were no signs of wasting syndrome (cancer cachexia), loss of appetite (anorexia), multiple tumor load that are mainly responsible for reduction in our research in body weight of cancer bearing animals. The relative lung weight was observed to be similar in different experimental groups after 22nd week study **Table 30**, this may be due to non-formation of multiple tumor nodules. However, in other studies due to formation of multiple tumors, a decline in body weight and growth in lung weight was observed (Mohan and Koul, 2018; Onami et al., 2017; Kamaraj et al., 2007; Kamaraj et al., 2009; Rajendran et al., 2008; Senthilnathan et al., 2006). Oral or intraperitoneal dose of B(a)P, that lead the formation of tumors showed growth in the lung weight of mice after study period (12 to 22 weeks) (Senthilnathan et al., 2006; Rajendran et al., 2008; Liu et al.,

2015). Increase in lung weights might be due to inflammation and tumor load in the mice treated with B(a)P (Manna et al., 2009). While the administration of different dietary infusions increases the body and decreases the lung weight of B(a)P given animals as compared to non-treated groups (Kamaraj et al., 2009; Ravichandran et al., 2011).

One of the biomarkers commonly used to assess the effectiveness of dietary substances is plasma antioxidant capacity (Boussoulim et al., 2016). Plasma total antioxidant capacity (TAC) usually decline in cancer, diabetes and atherosclerosis. The antioxidant system in plasma would gain useful information for health care (Kambayashi et al., 2009; Selvendiran and Sakthisekaran, 2004; Martin-Gallan et al., 2003; Santiago-Arteche et al., 2012). Total antioxidant capacity (TAC) is an important tool in estimating the endogenous antioxidant system of patients suffering from chronic diseases (Opara et al., 1999). The endogenous antioxidant system present in plasma includes catalase (CAT), glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD) and glutathione reductase (GR) (Codoner-Franch et al., 2010). There are no specific approaches for measuring the TAC but methods like 2,2'-azinobis [(3-ethylbenzthiazolinesulfonate)] (ABTS), the ferric reducing antioxidant power (FRAP), DPPH and total radical absorption potential (TRAP) can provide the information about the antioxidants present in the body fluids (Boussoulim et al., 2016; Moura-Nunes et al., 2009). DPPH and ABTS were found to be simple and reproducible approaches with regard to evaluating the antioxidant potential of various plant infusions (Sanchez-Moreno, 2002; Janaszewska and Bartosz, 2002). These chemical models were used previously to evaluate the plasma antioxidant activity in both humans and animals (Santiago-Arteche et al., 2012; Boussoulim et al., 2016). The B(a)P causes DNA damage by direct and indirect mechanisms through cytochrome P450 metabolism either by DNA adducts formation or by generating the ROS (Kasala et al., 2015). The oxidative stress that B(a)P induces in the blood and tissues can be measured by different methods such as determination of antioxidant potential of enzymes, estimation of protein carbonyl content, LPO, haemolysis and 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels (Koutelidakis et al., 2009; Sehgal et al., 2012). In B(a)P administered species, an rise in the lipid-peroxidation levels (serum, lung tissues and erythrocytes) and consequent decrease in activities of antioxidant enzymes that may be due to excessive ROS generation in the erythrocytes (Arivazhagan et al., 2004; Ravichandran et al., 2011; Asokkumar et al., 2012). In support to our study the plasma antioxidant potential was declined in B(a)P administered group (Gp.II) as compared to vehicle group (Gp. I) **Fig. 69** and **70**, which may be due to the model carcinogen B(a)P that interact with lipids of

membranes and leads the formation of free radicals (Sikkim et al., 2000). These radicals react with lipid molecules that cause lipid peroxidation which is directly related to formation of B[a]P-quinones (Pompella et al., 1991; Kim et al., 2000). These B(a)P-quinones influence the lipids and antioxidant enzymes present in blood suggesting a possible role of B(a)P-quinones in the carcinogenesis (Kim et al., 2000). The B(a)P treated animals demonstrated an increased level of TBARS (thiobarbituric acid reactive substances) in liver and lung tissues of mice (Venugopal et al., 2014). B(a)P also increases the oxidative damage and lipid peroxidation of different tissues of mice (Godschalk et al., 2003). SOD and CAT, the antioxidant enzymes were detected to be reduced in carcinogenic situations that might be due to increased LPO (Ramakrishnan et al., 2006).

The antioxidants present in dietary substances are known to have the ability to withstand the effects of oxidative stress by neutralizing the various harmful effects of free radicals that are formed in the body (Moskaug et al., 2005; Valko et al., 2006). GT, OG and GT+OG treated groups demonstrated an increase in radical scavenging potential as compared to animals administered with B(a)P **Fig. 69** and **70**. Increase may due to oral intake of herbal extracts that undergo gastrointestinal digestion followed by absorption in blood where they employ antioxidant effects and may reduce the oxidative damage (Koutelidakis et al., 2009). Green tea is abundant in polyphenols, mainly flavonoids, which may disrupt the intake of some endogenous antioxidants in the human body (Lotitio and Fraga, 1998; Koch et al., 2018). Soy and GT alone or in combination have been documented to increase the total antioxidant ability of hypercholesterolemic patients, while total cholesterol levels have only decreased (de Santana et al., 2008). The GT combination and vitamin E demonstrated a significant upsurge in endogenous antioxidants and body weight as compared to isoproterenol, green tea or vitamin E alone treated groups (Upaganlawar et al., 2009). The animals receiving the infusion of green tea and *Pelargonium purpureum* augmented the antioxidant status of blood plasma, heart and lung homogenates as compared to white tea (Koutelidakis et al., 2009). The clinical investigations revealed that administration of black and green teas significantly boosts the ABTS and FRAP plasma antioxidant activity in humans (Leenen et al., 2000; Sung et al., 2000). The aqueous infusion of *O. gratissimum* demonstrated an increase in both plasma and hepatic CAT and SOD activities ($p < 0.05$) but no changes in plasma and liver protein concentrations as compared to animals of control group (Ighodaro and Ebuehi, 2009). The enhancement in plasma antioxidant capacity against free radicals may be due to

the increased endogenous antioxidant levels attained by the treatment of GT, OG and GT+OG infusions.

The degree of neoplastic pathological alterations was significantly higher in Gp. II animals **Fig.73**. It was reported that B(a)P- induced lung histological changes are associated with pro-apoptotic gene down-regulation (p53, Bax and Caspase), anti-apoptotic gene (Bcl2) and growth promoting gene up-regulation (Hras and cMyc), that plays part in lung tumor initiation (Meuwissen and Berns, 2005; Banerjee et al., 2006; Manna et al., 2006). Experimental evidence exposed that during carcinogenesis induced by benzo(a)pyrene in mice showed different cellular and histopathological changes that are categorized as hyperplasia, dysplasia, carcinoma in situ and invasive carcinoma (Estensen et al., 2004; Manna et al., 2009). Present investigation revealed that benzo(a)pyrene-induced animal (Gp.II) showed a progressive histopathological alterations that were identified as hyperplasia and adenoma (in only 2 mice of Gp. II) as early lesions of lung carcinogenesis after 22th week of benzo(a)pyrene administration. These alterations in the histopathology of lungs may be due to change in the expression of certain genes. Earlier studies also showed that B(a)P treatment revealed a regular augment in lung lesions over and done with inflammation, hyperplasia, dysplasia and carcinoma in situ (Banerjee et al., 2006; Manna et al., 2006).

There were no multiple tumor formation in this study, that may be due to less susceptibility of mice strain (Swiss albino mice) to benzo(a)pyrene, concentration of the dose, multiple doses, age of the mice, route of administration. Some reports showed that a single or multiple intraperitoneal doses of B(a)P with dose ranging from 50-100mg/Kg B.W in A/J or Balb/c mice demonstrated tumor formation after different time period (22-26 weeks) (Yan et al., 2005; Yan et al., 2006; Yeo et al., 2017; Mohan and Koul, 2018). Oral intubation of B(a)P given at multiple times at 2-50mg/Kg B.W in Swiss albino mice leads the formation of lung tumors after 16th and 28th weeks of first dose of carcinogen (Katiyar et al., 1993; Kamaraj et al., 2009; Venugopal et al., 2014). While as some studies demonstrated that single subcutaneous injection of B(a)P of 0.02- 0.2mg was administered at sub-scapular region of strain A mice revealed no nodule or tumor formation but histopathological changes were observed in the lung tissues at 8th, 17th, 26th and 36th week of B(a)P administration (Banerjee et al., 2006; Manna et al., 2009). However, a solo intraperitoneal dose of B(a)P at 0.25, 0.50 and 1 mg/head in A/J mice for 26 weeks showed concentration dependent hyperplastic changes but no malignant neoplasm (Onami et al., 2017).

The degree of histopathological lung lesions were significantly reduced in GT (Gp. III), OG (Gp. IV) and GT+OG (Gp. V) treated animals as compared to Gp. II animals **Table 32**. This inhibition in hyperplastic lesions by GT, OG and GT+OG extracts may be due to modulation of antioxidant enzyme status, expression of oncogenes and tumor suppressor genes. Green tea and *O.gratissimum* treatments demonstrated preventive effects by different mechanisms including variation in carcinogen-metabolizing enzymes and cell-signaling pathways, Cell-cycle arrest and apoptosis induction has suppressed the activation of transcription factors that cause cancer progression to decrease (Rajeshwari, 1992).

The treatment of green tea catechins has shown that downregulation in the expression of growth-regulating genes (H-ras, c-myc, cyclin D1) and anti-apoptotic genes (Bcl-2, p21) and upregulation of pro-apoptotic genes (p53, Bax and p27) helps to minimize lung cancer (Manna et al., 2009). The cell viability of A549 cells resulting from the stimulation of apoptotic signaling and reduction of anti-apoptotic signaling was repressed by the *O. gratissimum* extract, indicating that *O. gratissimum* extract can be useful for lung cancer treatment (Chen et al., 2010). The animals having lung carcinoma demonstrated a significant decrease in the tumor size by the ethanolic extract of *Ocimum sanctum* (Rajeshwari, 1992). It is believed that diets supplemented with single component do not revealed the same health properties as that of the whole food (Liu et al., 2003). In current study GT, OG and GT+OG infusions were found equally effective against the reduction of B(a)P -induced early lung lesions **Table 32**. Phytochemicals of the binary mixture may act differently in *in vivo* conditions, GT and OG may act in such a way that they normalize the activity in GT+OG binary combination. As a result of various pathways such as metabolism, activity of detoxifying enzymes, altering growth factors and response to immune system, the phenolic compounds act differently *in vivo* conditions (Yi et al., 2005).

The treatment of GT, OG and GT+OG decreased the occurrence of hyperplasia compared to Gp. II. The alveolar, bronchiolar and alveolar/bronchiolar hyperplastic zones were also found to be reduced and no adenoma formation in the treatment groups (III, IV and V) compared to animals given B(a)P. However, all infusions (GT, OG and GT+OG) displayed similar ($p \geq 0.05$) reduction in hyperplastic zones as compared to B(a)P administered group (Gp. II) **Table 32**. The reduction in hyperplastic regions lung lesion might be due to increased bcl-2 expression at both RNA and protein levels (Manna et al., 2009). The histopathological alterations are the early signs of carcinogenesis and the mentioned infusions suggest a protective role against carcinogenesis process and decrease the occurrence of early lesions. Thus green tea, *O. gratissimum* and their combination (GT+ OG) demonstrated a preventive

effect against B(a)P induced changes. Green tea showed its chemopreventive potential in many animal models (Zhang et al., 2000; Liao et al., 2004). The green tea, green tea polyphenols and its combination with drugs or other herbs can inhibit carcinogenesis when given during initiation, postinitiation or during entire experiment (Lambert, 2013). Compared to control and treatment with aerosolized difluoromethylornithine (DFMO), Poly E inhibited the pressure of lung cancer, there was no major effect on tumour growth compared with aerosol control. While the combination of aerosolized DFMO and dietary Poly E did not have increased the efficacy compared with either agent alone (Anderson et al., 2008). Green tea and quercetin combination displayed increased reduction in prostate cancer xenograft tumor growth as compared to green tea and quercetin alone. This is related to increased bioavailability of non-methylated GTP that leads to enhanced antiproliferative and pro-apoptotic effect (Wang et al., 2014). *In vivo* study, benzo(a)pyrene induced tumor multiplicity was observed to be reduced by polyphenon E treatment, however failed to prevent lung carcinogenesis by polyphenon E without EGCG at the same dose (Fu et al., 2009). The curcumin combination with (-)-epicatechin (EC) extensively increased the inhibition of cell growth in PC-9 (human non-small cell lung carcinoma cell line) compared with curcumin or EC alone (Saha et al., 2010). The *In vitro* studies displayed that combination of green tea with its own phytochemicals, other herbs and drugs demonstrated more activity as compared to individual extract (Saha et al., 2010; Fujiki et al., 2015).

The current study revealed that administration of GT+OG showed the highest plasma DPPH and ABTS quenching ability followed by GT and OG as compared to B(a)P treated group. Although histopathological examination of lung tissue demonstrated that GT, OG and GT+OG showed the similar reduction in alveolar, bronchiolar and alveolar/bronchiolar hyperplastic zones. Hence GT, OG and GT+OG may be considered as a plausible chemopreventive agent against the progression of respiratory tumorigenesis.

CHAPTER 8
SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

Green tea, herbal teas (*Ocimum gratissimum*, *Cymbopogon citratus*, *Cymbopogon flexuosus* and *Hibiscus-rosa sinensis*) and the binary combination of green tea with herbal teas were evaluated for some biological activities. GT and GT combinations illustrated higher radical quenching abilities as compared to single plant extracts/ individual herbal teas in chemical (DPPH, ABTS and NO) and ex vivo models (LPO and haemolysis). The antioxidant interactions were determined on the basis of dose effect relationship employing Compusyn software. Among different GT/herb pair, additive to synergistic interactions were observed. The single infusion of GT revealed highest TPC and TFC in comparison to GTCs and other individual infusions. However, the experimental value of TPC/TFC was found to be lower than the theoretical value for all binary mixtures except a slight increment in experimental value was noticed for GT+CF combination and no difference in flavonoid content of GT+CC combination. A high negative correlation was observed between TPC/TFC and antioxidant activities (EC_{50}) of individual infusions except for anti-lipid peroxidation activity. In case of GTCs, negligible to high correlation was observed between TPC/TFC and antioxidant activities (EC_{50}).

The ATR-FTIR analysis of infusions for MIR (mid infrared region, $4000-600\text{cm}^{-1}$) denoted multiple peaks. All individual infusions manifested five major absorption regions with peaks of varying intensity and width. The most prominent peak observed was in the range of $3000-3600\text{cm}^{-1}$. This peak indicated the overlapping of hydroxyl group of alcohols or phenols, C-H stretch of alkene, arenes and N-H stretch of amine groups. This region was also selected for correlation study between FTIR peak intensity (%T) and TPC/antioxidant activity (EC_{50}). GT combinations demonstrated similar spectra, as that of GT single infusion except few additional peaks in the region $3600-4000\text{cm}^{-1}$. GT+OG demonstrated the most intense peak in region $3000-3600\text{cm}^{-1}$ followed by GT+HR, GT+CF and GT+CC. A correlation study was conducted between FTIR peak intensity ($3000-3600\text{cm}^{-1}$) and TPC/ EC_{50} of different samples. For individual infusions, a very high negative correlation was observed between TPC and FTIR peak ($3258-3283\text{cm}^{-1}$) and very high positive to moderate positive for antioxidant activity and FTIR except LPO assay (negligible correlation). However, for GTCs a negligible correlation was noticed between TPC and FTIR peak ($3206-3267\text{cm}^{-1}$) and very high positive to low correlation between antioxidant activity and FTIR. FTIR selected peak intensity was found to be a better tool for predicting radical scavenging activity of GT combinations. Moreover, there is a need to develop and employ advanced chemometrics on FTIR data that

may provide more relevant information for indicating radical scavenging potential. UV- VIS analysis revealed that among all individual infusions (GT, OG, CC, CF and HR), GT demonstrated an intense peak in the region between 250nm-300nm and other infusions showed a moderate to low absorption in this region, whereas this peak was found in all GTCs. The UV-VIS region (250-300nm) was also selected for correlation study between absorbance and TPC/antioxidant activity (EC_{50}), because the peak of catechins mostly EGCG (major component of green tea) and caffeine was observed in the same region. For single infusions, a high positive correlation was detected between TPC and absorbance (272nm) and a very high negative to high negative correlation for antioxidant activity and absorbance except LPO assay (negligible correlation). Although for GTCs, moderate positive correlation was found for TPC and absorbance (272nm) and high negative to low correlation for antioxidant potential and absorbance except ABTS and LPO (negligible correlation). Among all these methods (TPC/TFC, FTIR and UV) performed, the TPC is better method to tell us about the correlation with antioxidant activity. But it is not necessary that only on the basis of TPC; one can observe the antioxidant potential. It needs to be evaluated by other advanced methods.

The anti-angiogenic potential of all infusions (GT, OG, CC, CF, HR, GT+OG, GT+CC, GT+CF and GT+HR) was investigated against VEGF-induced angiogenesis using CAM model. The *in vivo* CAM model illustrated that all infusions reduced the new vascularization but GT and GT+OG combination displayed highest anti-angiogenic potential. Overall, GT and OG combination illustrated highest antioxidant and anti-angiogenic potential.

The proportion of individual herb can play a vital role in determining the chemopreventive potential or bioefficacy of herbal combination. This combination (GT and OG) at different ratios (3:1, 2:1, 1:1, 1:2 and 1:3) was further scrutinized. It was observed that green tea and *O. gratissimum* combinations displayed significant antioxidant potential at their equal (1:1) proportion, showing the highest radical quenching ability and strongest synergism in chemical and *ex vivo* models. Among studied binary combinations GT showed the strong and broad peak in the region of $3000-3600\text{ cm}^{-1}$ followed by 3:1, OG, 1:1, 2:1, 1:2, and 1:3, respectively. They showed similar spectra as that of individual samples with difference in broadness and intensities of peaks and a slight shifting in the wavenumbers. Green tea demonstrated the highest absorption peak followed by combination 3:1, 1:1, 2:1, 1:2, 1:3 and very low absorption peak in *O.gratissimum*. A high negative correlation was observed between TPC and FTIR peak intensity while as negligible correlation between antioxidant

activity and peak intensity of different ratios. For UV analysis a low correlation was detected between TPC and absorbance (272nm) and moderate to high correlation for antioxidant activity and absorbance.

The GT+OG combination (1:1) was further screened for cytotoxicity effect against A549 cell lines. All samples GT, OG and binary mixture GT+OG showed cytotoxicity effect in a dose-independent manner. This combination was also tested for cancer chemopreventive effect in vivo against B(a)P - mediated lung lesions in mice model. The oral administration of GT+OG combination demonstrated highest plasma DPPH and ABTS scavenging potential as compared to individual infusions (GT and OG). B(a)P induced animals demonstrated distorted architecture and presence of hyperplastic zones. However, GT, OG and GT+OG treated animals demonstrated similar reduction in these hyperplastic regions (Gp. III, Gp. IV and Gp. V animals) than the Gp. II.

The administration of GT+OG at equal proportion showed the high plasma DPPH and ABTS quenching ability followed by GT and OG as compared to the B(a)P treated group. The histopathological examination of lung tissue demonstrated that GT, OG and GT+OG showed the similar reduction in alveolar, bronchiolar and alveolar/bronchiolar hyperplastic zones. Hence GT, OG and GT+OG may act a plausible chemopreventive agent against lung tumorigenesis. This study provides a scientific basis for designing and formulating beverages containing GT combinations based on their antioxidant interactions that can potentially enhance the efficacy of GT as a chemopreventive agent.

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