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Anti-Cancer Role of *Cassia alata* Leaf Extract in a Rat Model of Lung Cancer

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Abstract: Lung cancer was diagnosed in 2.09 million people and killed 1.76 million globally in 2018, making it the leading cause of cancer-related mortality in men and the second leading cause in women. Rats were categorized into four groups (10 rats/each), including Control (CTRL) group, the Ethyl carbamate (EC) group, the Ethyl carbamate+ *Cassia alata* leaves extract (EC+CALE) treated group, and the Ethyl carbamate + Cisplatin (EC+CIS) treated group. Lung index estimation, histological, biochemical, and molecular assays were done. The lung index was significantly increased in the EC group, but after treatment with CALE, the lung index decreased. Hematoxylin & Eosin stain showed the development of lung cancerous lesions in the epithelial lining of terminal bronchioles, CALE treated group provided more or less restoration of normal lung histology with no tumor mass observed. Also, CALE treatment restored the changes in glutathione, superoxide dismutase, malondialdehyde, and wild-type epidermal growth factor receptor (EGFR) - mRNA expression levels in lung tissues after treatment of lung cancer-bearing rats with CALE. The current study showed that *Cassia alata* leaves extract could be used as a new source of natural agents fighting lung cancer.
Keywords: *S*

1. Introduction

Lung cancer represents roughly 4.6%, including all cancers for both genders in Egypt, accounting for approximately 6.9% of men cancers (5017 cases) and 2.5% of women cancers (3634 cases), with a mortality rate approaching 6.8% in men and 3.8% in women. In 2002, the death rate from lung cancer in Egypt was 4,429 per 100,000 persons [1]. Lung cancer was diagnosed in 2.09 million people. It killed 1.76 million people globally in 2018, making it the leading cause of cancer-related mortality in men and the second leading cause in women [2]. Histologically, non-small cell lung cancer (NSCLC) accounts for roughly 85%, and small cell lung cancer (SCLC) accounts for approximately 15% of all lung malignancies are the two main categories of lung cancer. Surgery, chemotherapy, radiation, and immunotherapy are currently available treatments. The high incidence and prevalence of lung cancer could provide a platform for new insights into the impact of public health interventions, genetics, and novel treatment options [3-5]. NSCLC accounts for around 85% of lung malignancies and is divided into lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), as well as large-cell carcinoma (LCC) [6].

Ethyl carbamate (EC), urethane is a carbamic acid ester. It was discovered mainly as a byproduct of fermented foods and beverages. Because of inadvertent fermentation during the manufacturing or storage of fermented foods and beverages, EC is extensively spread [7]. Ethyl carbamate (EC) is a chemical carcinogen (IARC group 2A). Because of the development of an active metabolite, vinyl carbamate, EC caused malignant lesions in several locations. Repeated exposure to ethyl carbamate found in fermented foods and beverages increases the risk of lung cancer by causing irreversible mitochondrial malfunction and increasing cellular mitotic activity [8].

Platinum drugs (e.g., cisplatin carboplatin), taxanes (e.g., paclitaxel, docetaxel), vinorelbine, etoposide, pemetrexed, and gemcitabine are among the chemotherapy regimens recommended based on performance status [9]. Platinum-based chemotherapy is still the mainstay of care for most patients with advanced non-small cell lung cancer (NSCLC) [1]. The excessive toxicity and chemoresistance associated with traditional chemotherapies reduce the success rates of the current chemotherapeutic regimen, necessitating the development of a more efficient and safer alternative treatment strategy [10].

Numerous studies indicate that natural polyphenols have chemo-preventive and/or chemotherapeutic capabilities against various forms of cancer by acting through various molecular pathways [11]. Phytochemicals, which are naturally occurring plant components, are a significant source of novel drugs as well as sources of treatment for cancer. Taxol analogs, vinca alkaloids like vincristine and vinblastine, and podophyllotoxin analogs are common examples. These phytochemicals frequently function by influencing molecular pathways implicated in cancer development and progression. Specific pathways include increased antioxidant status, carcinogen inactivation, inhibition of proliferation, promotion of cell cycle arrest and death, and immune system control [12]. The Fabaceae family is the second most widespread family in the plant kingdom, with species found worldwide. The Fabaceae family contains a high concentration of phytochemicals (flavonoids, phenolic acids, saponins, alkaloids, lectins, and carotenoids) which have a variety of health benefits, notably anti-cancer activities [13]. Fabaceae as Fava beans restrictions for people

with glucose-6-phosphate dehydrogenase deficiency are historically known to provoke severe acute hemolytic anemia in individuals with G6PD deficiency [14].

Senna alata is known as *Cassia alata*. It is an essential medicinal shrub in the family Fabaceae, order Fabales. It is known by several common names including candle bush, winged senna, Christmas bush, ringworm shrub, and king of the forest, among others. It is widely used in traditional medical practice in many developing countries [15]. Among the reported chemical components in *Cassia alata* are phenols (chrysaphanol, rhein, aloe emodin, kaempferol, and glycosides), anthraquinones (alatonal and alatinone), fatty acids (palmitic, oleic and linoleic acids), steroids, and terpenoids (stigmasterol, sitosterol, and campesterol) [16]. These secondary metabolites perform a variety of biological functions [17]. The plant leaves have been proven to possess phenolics and to have cytotoxic activity against cancer cells. Chrysoeriol, kaempferol, quercetin, palmitic acid, stearic acid, and other major chemical constituents are present. Most of these chemicals have been shown to perform anti-inflammatory and cytotoxic activity against lung cancer [18, 19].

The present study aims to evaluate the anticancer role of Cassia alata leaves extract (CALE) against Ethyl carbamate-induced lung cancer compared to the Cisplatin drug.

2. Materials and method

2.1. Chemicals

Ethyl Carbamate, commonly known as urethane, 99.0% (GC), A Sigma-Aldrich product. 1 vial of Cisplatin 50 mg/ 50 ml (Mylan) was purchased from Elezaby pharmacy, Cairo, Egypt. Bio-diagnostic Company provided the oxidative stress kits in Cairo, Egypt. Other chemicals purchased from El-Gomhoria Company, Egypt, were of analytical grade.

2.2. Preparation of *Cassia alata* Leaves Extract (CALE)

Cassia alata leaves were collected from the Faculty of Agriculture farm at Cairo University and kindly identified by Dr. Mona Marzok, Taxonomist at the National Research Centre Herbarium in Cairo, Egypt. Fresh leaves were dried in the shade and ground to a fine powder at room temperature. Methanol was employed to extract the air-dried, powdered (2 kg) material, which was then concentrated under reduced pressure at 40o C. The methanolic extract residue (22.57 percent) was concentrated to dryness in rotor vapor at 40 °C. The extracted materials were kept at -20 °C until they were used [20].

2.3. Phytochemical analysis of CALE

CALE was exposed to Fourier transform infrared spectrophotometric (FTIR) analysis according to Pakkirisamy et al. [21], where the scanning range for transmittance was 4000 cm-1 to 400 cm-1 (mid-IR region).

2.4. Experimental animals and design

At the start of the experiment, forty adult male albino rats weighing 150±20 g were acquired from the New Veterinary Office in Giza, Egypt. Rats were kept in wire cages in a temperature-controlled environment with 12-hour light/dark cycles. Food and water were freely available during the experiment. All experiments were approved by the Institutional Animal Animal Care and Use Committee at Helwan University's Zoology Department, Faculty of Science (Approval number: HU/IACUC/Z/MI1101-23).

The rats were categorized into four groups (10 rats/ group) represented in (Figure 1) as the following:

Group 1- Control (CTRL) group: Rats were intra-peritoneally injected with phosphate-buffered saline (PBS), PH =7, daily for 7 months.

Group 2- EC group: For lung cancer induction, 30rats were intra-peritoneally injected with ethyl carbamate solution 1g/kg/rat once weekly for a month (Sigma-Aldrich, USA) prepared by dissolving ethyl carbamate in PBS at 100 mg/ml [22]. Lung cancer developed after 5 months of latency from the first urethane injection. 10 rats were left untreated for the next two months representing this group.

Group 3- EC+CALE treated group: ten rats were orally administered with CALE (500 mg/kg/day) for the last 2 months [23].

Group 4- EC+CIS treated group: ten rats were intra-peritoneally injected with cisplatin (2.5 mg/kg) weekly for the last 2 months [24].

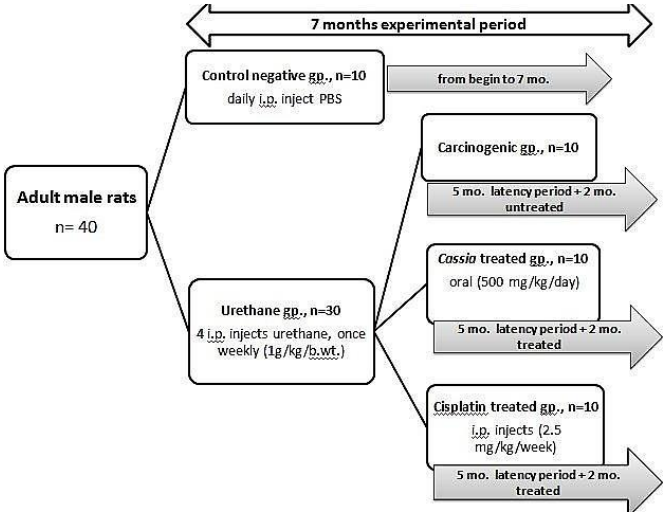

Figure 1. Graphical representation of the experimental design, including groups and ways of treatments.

2.5. Sample collection

Rats were sacrificed under 6% isoflurane anesthesia, and their lungs were immediately removed, cleaned in saline, blotted dry, and weighed. Each removed lung was split into three portions; the first was preserved in 10% neutral-buffered formalin for histopathology, while the second was kept at -20 °C for biochemical analyses. The third portion of the lung tissue was frozen at 80 °C for molecular analysis to estimate EGRF mRNA expression by quantitative real-time polymerase chain reaction (QPCR).

2.6. Lung index measurement

Five rats' lung and body weights for each group were weighed using a digital scale to calculate the lung index. Using the formula: lung index (percent) = (Recorded lung weight / Final body weight) x 100.

2.7. Histopathological analysis

A piece of the lung from each group was preserved in 10% phosphate-buffered formaldehyde. The fixed specimens were prepared for paraffin sectioning. Serial sections (5 microns) were produced [25] and stained with hematoxylin and eosin for general histopathological examination of tissue and cells [26]. After staining, the slides were viewed using a light microscope (LEICA model) and photographed with a Zeiss camera.

28. Biochemical analysis

Lungs were homogenized in 5–10 ml cold buffer (50 mM potassium phosphate, pH 7.5) per gram of tissue. Centrifugation was carried out at 4°C for 15 minutes at 2000 ×g and the produced supernatant was stored at -80°C for further analysis. Reduced glutathione concentration, superoxide dismutase activity, and malondialdehyde content in lung homogenate were determined using the analytical methods of Beutler et al.; [27], Nishikimi et al.; [28] and Ohkawa et al.; [29], respectively.

2.9. Quantitative real-time Polymerase Chain Reaction (QPCR)

2.9.1. Isolation of total RNA:

Total RNA was isolated from the lungs of the study groups using the RNeasy Mini Kit (Qiagen, Germantown, MD, USA) and kept at -80 °C according to the manufacturer's instructions. A Beckman dual spectrophotometer was used to determine the concentration of extracted RNA (Beckman Instruments, Ramsey, MN, USA).

qPCR was used to examine the expression level of the wild-type EGFR gene. The EGFR primer sequence (F: 5'-GAGAGGAGAACTGCCAGAA-3') & R: 5'GTAGCATTTATGGAGAGTG-3') [30], while primer sequence of the housekeeping gene, B-actin, (F: 5' GGCACCACACCTTCTACAATG-3'& (5'GGGGTGTTGAAGGTCT CA AAC-3') [31].

2.9.2. Complementary DNA (cDNA) synthesis:

Using a high-capacity cDNA reverse transcriptase kit, 1 µg of total RNA was extracted from each sample to synthesize cDNA (Applied Biosystems, Thermo Fischer Scientific, USA).

2.9.3. Quantitative polymerase chain reaction:

The cDNA was then amplified using the SYBR Green I PCR master kit (Thermo Fisher Scientific Inc., Lithuania) and the Step One apparatus (Applied Biosystems, Thermo Fischer Scientific), as follows: The enzyme was activated for 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C for denaturation, 20 seconds at 55°C for annealing, and 30 seconds at 72°C for an extension.

The ΔΔCT method was used to normalize changes in target gene expression relative to the mean critical threshold (CT) values of b-actin, the housekeeping gene. Fold change was calculated as a relative quantitation using the $2^{-\Delta\Delta Ct}$ method [32]. For each sample, the Ct value of the target gene (EGFR) mRNA was normalized against the B-actin endogenous control as ΔCT ($\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{B-actin}}$). The fold change of the target gene mRNA in the experimental sample relative to the control sample was determined by $2^{-\Delta\Delta Ct}$, where $\Delta\Delta CT = \Delta Ct_{\text{Experimental}} - \Delta Ct_{\text{Control.}}$

2.10. Statistical analysis:

A one-way ANOVA, according to equality of variance, was used to identify statistical differences between groups. Furthermore, the post-hoc tukey's test was performed for multiple comparisons between the experimental groups. The differences were considered significant at $p < 0.05$. All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) version 25.

3. Results

3.1. Fourier transform infrared spectrophotometric analysis

This analysis was necessary because no previous FTIR analysis had been done for this extract, specifically the methanolic extract of CALE from the Egyptian cultivar. FTIR spectrum analysis of CALE is presented in (Table 1, Figure 2). CALE contained different groups like OH, NH, C-H, C-N, N-O, C-Cl, C=C, C=C=C, C=O, C=N, S=O, N=N=N, N=C=S, C≡C, S-C N∈Saturated and unsaturated hydrocarbons, alcohols, carbonyl compounds as carboxylic acids and conjugated acids, amines, nitro- compound, halo-compound, and thiocyanates and isothiocyanates were the expected compounds present in CALE.

Table 1. Fourier transform infrared spectrophotometric analysis of *Cassia alata* leaves extract

Absorption (cm^{-1})	Appearance	Group	Compound Class
3339.88	Strong broad	O-H…	Alcohol
2957.54	weak	C-H stretching	alkene
2928.52	medium	C-H stretching	alkane
2851.62	medium	C-H stretching	alkane
2371.04	weak	-	-
2202.72	weak	CΞC stretching	alkyne
2166.07	weak	S-CΞN stretching	thiocyanate
2147.56	weak	N=N=N stretching	azide
2093.46	weak	N=C=S stretching	isothiocyanate
2008.71	weak	N=C=S stretching	isothiocyanate
1993.85	weak	C=C=C stretching	allene
1979.26	weak	C=C=C stretching	allene
1952.20	weak	C=C=C stretching	allene
1735.19	medium	C=O stretching	carboxylic acid
1655.09	weak	C=O stretching	conjugated acid
1626.17	weak	C=N stretching	Imine/oxime
1459.92	medium	N-O stretching	nitro compound
1377.08	strong	O-H bending	carboxylic acid
1243.46	medium	C-N stretching	amine
1036.28	strong	S=O stretching	sulfoxide
755.08	strong	C-Cl stretching	halo compound

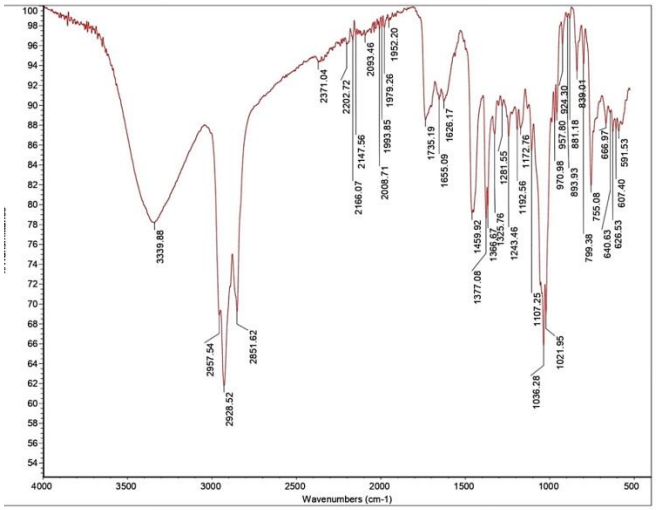

Figure 2. Fourier transform infrared spectrophotometric analysis of *Cassia alata* leaves extract

3.2. Lung index

Lung index was significantly increased in the EC group as compared to the CTRL group. EC+CALE and EC+CIS treated groups showed a significant decrease in lung index as compared to the EC group (Figure 3).

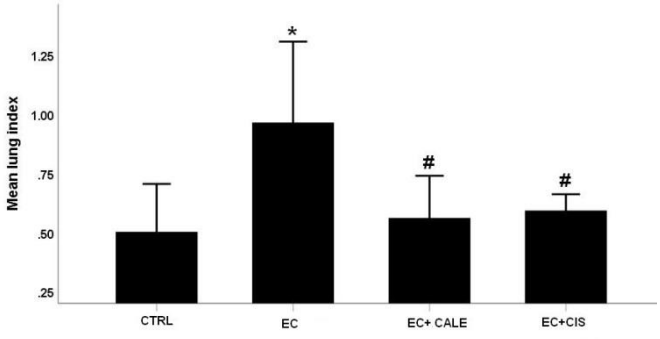

Figure 3. Mean lung index among different groups. *: significant change at P < 0.05 against CTRL group, #: significant change at P < 0.05 against ethyl carbamate (EC) group.

3.3. Histopathology

CTRL group showed normal lung histology including terminal bronchiole, respiratory bronchiole, alveolar sac, spongy-shaped alveoli, thin inter-alveolar septum, and thick interstitial tissue (Figure 4A). EC group showed lung adenocarcinoma appeared as a well-defined tumor mass formed of irregular papillae of the terminal bronchiole`s epithelial linning supported by hypertrophied discontinuous muscle fiber layer and surrounded by peri-bronchiolar inflammation. Lung parenchyma appeared with collapsed alveoli and thickened interstitial tissue (Figure 4B). EC+CALE treated group showed the absence of tumor mass and almost restoration of normal lung histology with dilated blood vessels (Figure 4C). EC+CIS treated group showed the absence of tumor mass but with some histologic lesions such as peri-bronchiolar inflammation, extravasation of RBCs in alveolar space, and dilated blood vessels (Figure 4D).

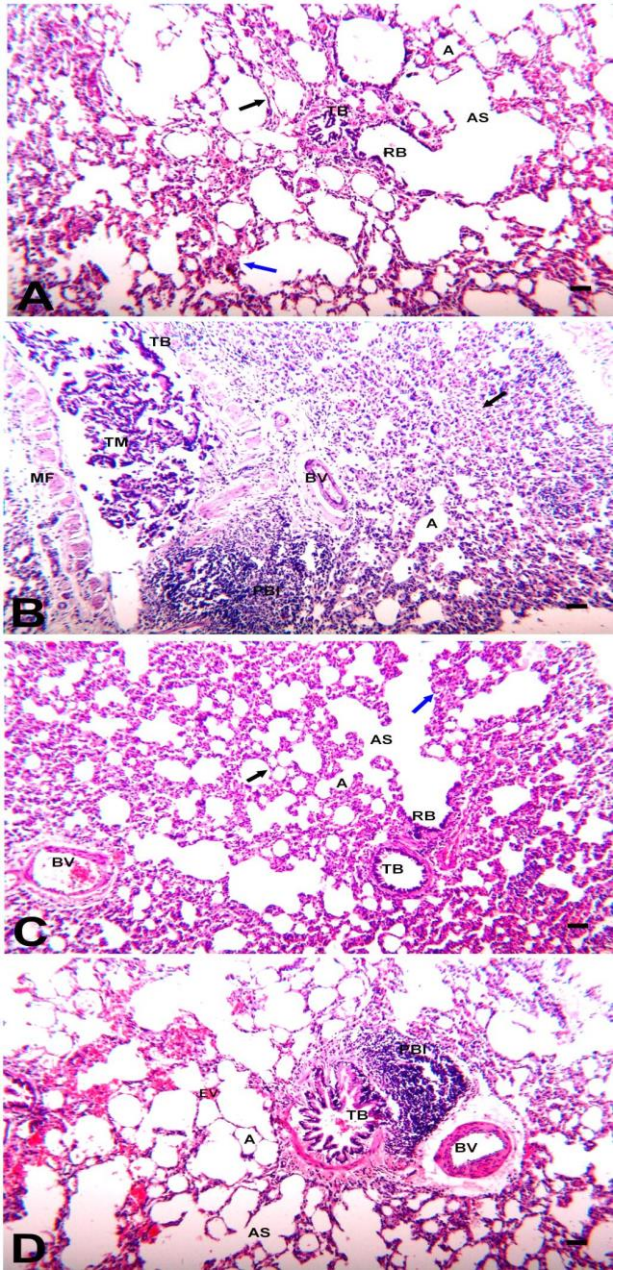

Figure 4. A transverse section in the lung of different groups stained by H&E stain (100×). (A) CTRL group showed normal histo-architecture of the lung, including terminal bronchiole (TB), respiratory bronchiole (RB), alveolar sac (AS), spongy-shaped alveoli (A), thin inter-alveolar septum (black arrow) and thick interstitial tissue (blue arrow). (B) EC group showed lung adenocarcinoma exhibited a well-defined tumor mass (TM) formed of irregular papillae formed from the epithelial lining of (TB) which was supported by hypertrophied discontinuous muscle fiber (MF) layer and surrounded by peri-bronchiolar inflammation (PBI). Noticeable thickening of interstitial tissue (black arrow), and collapsed alveoli (A) were noted. (C) EC+CALE treated group showed almost restoration of normal lung histology including (TB), (RP), (AS), spongy-shaped (A), thin inter-alveolar septum (black arrow) and thick interstitial tissue (blue arrow), with dilated blood vessel (BV). (D) EC+CIS treated group showed folded (TB) surrounded by peri-bronchiolar inflammation (PBI), (AS), (A), extravasation (EV) of RBCs in alveolar space, and dilated blood vessel (BV). Bar =50 micrometers.

3.4. Biochemical results

The statistical comparisons of GSH concentration, SOD activity, and MDA content among different study groups showed in (Table 2, Figure 5). Glutathione concentration was significantly decreased (P< 0.05) in the EC group compared to the CTRL group. Rats of the EC+CALE group and the EC+CIS treated groups showed a significant increase (P< 0.05) as compared to the EC group (Figure 5A). Superoxide dismutase was significantly decreased (P< 0.05) in the EC group compared to the CTRL group. Rats of the EC+CALE group and EC+CIS treated groups showed a significant increase (P< 0.05) as compared with the EC group (Figure 5B). Malondialdehyde content was significantly increased (P< 0.05) in the EC group compared to the CTRL group. Rats of the EC+CALE group and EC+CIS treated groups showed a significant decrease (P < 0.05) as compared with the EC group (Figure 5C).

Table 2. Biochemical results of GSH concentration, SOD activity, and MDA content in lungs of the study groups. All data expressed as Mean ± S.D.

Parameter / Group	GSH Conc. (mg/gram tissue)	SOD Activity (U/gram tissue)	MDA content (nmol./gram tissue)
CTRL	3.7833± 0.86558	921.00± 61.098	97.67± 14.012
EC	1.8933± 0.208 *	487.67± 26.312 *	450.00± 66.566 *
EC + CALE	3.7200± 0.515 #	912.00± 16.823 #	155.33± 25.027 #
EC + CIS	3.6633 ± 0.675 #	898.00± 25.060 #	179.00± 15.111#

3.5. Result of QPCR

Gene expression level of EGFR mRNA was significantly decreased (P < 0.05) in the EC group compared with the CTRL group. Rats of EC+CALE and EC+CIS treated groups showed a significant increase in EGFR gene expression (P < 0.05) as compared with the EC group (Figure 6). This result highlighted the critical role of EGFR expression in lung cancer incidence. EGFR expression was decreased or down-regulated in this type of ethyl carbamate-induced lung cancer. The role of *Cassia alata* leaves extract was marked in regulating the changes of EGFR expression level almost to its normal level.

4. Discussion

In developed and developing countries, lung cancer contributes significantly to morbidity, mortality, and medical expenses. In the current study, EC dramatically increased lung weight and index due to inflammatory cell aggregation and increased proliferation of malignant cells in the lungs [33]. According to Petruzzelli and Wagner [34], cancer cachexia could explain such a reduction in body weight. The degradation of adipose and skeletal tissue in the host body exposed to a carcinogen may result in a constant loss of body weight. The EC+CALE treated group had a substantial decrease in lung weight and relative lung weight compared to the EC group in the current investigation.

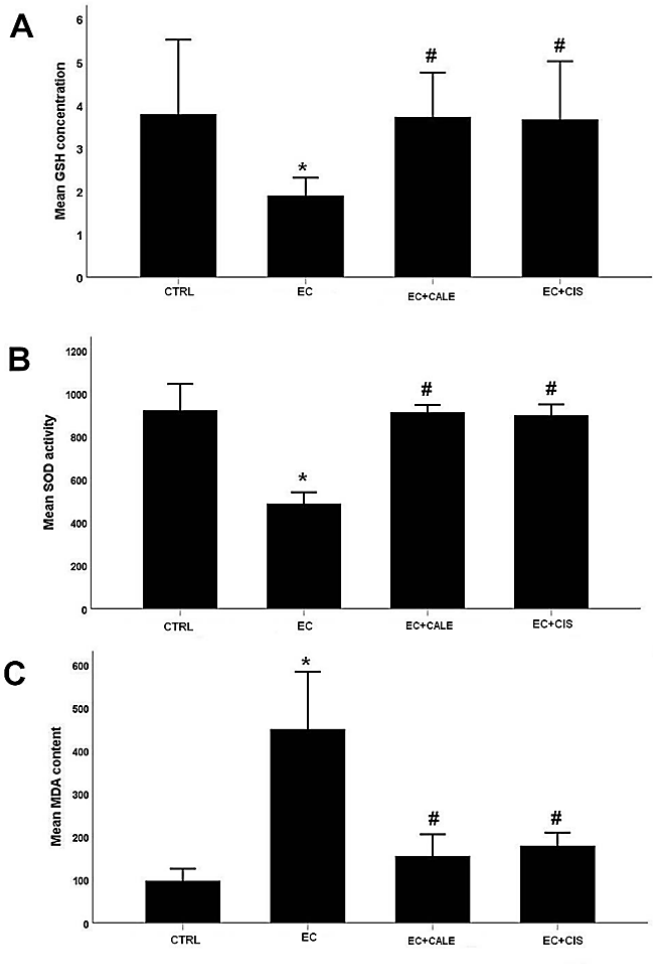

Figure 5. Biochemical results of (A) Glutathione (GSH) concentration, (B) Superoxide dismutase (SOD) activity, and (C) Malondialdehyde (MDA) content in the study groups. *: significant change at P < 0.05 against CTRL group, #: significant change at P < 0.05 against EC group.

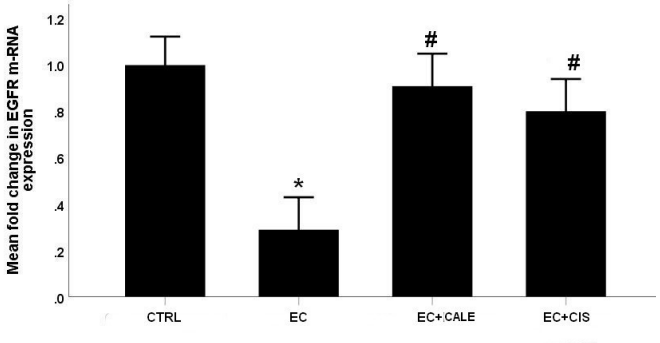

Figure 6. Mean fold change of EGFR mRNA expression in the lung of the study groups. *: significant change at P < 0.05 against CTRL group, #: significant change at P < 0.05 against ethyl carbamate (EC) group.

The terminal bronchiole lumen was invaded and destroyed by a tumor mass in the EC group's infiltrative bronchiolar adenocarcinoma. A mixture of pleomorphic cells with various cell sizes and shapes and hyperplastic columnar epithelial cells with basal vesicular nuclei make up the tumor mass. In agreement with the findings of the present investigation, Stakisaitis et al. [35] noted that the nuclei of bronchiolar adenocarcinoma cells were pleomorphic cells with frequent mitosis, tumor cell infiltration into the bronchiolar lumen, and wall push on the surrounding tissues.

Our lung cancer model was similar to that of Radwan et al. [36], who used four intra-peritoneal injections of EC at a dosage of 0.375 g/kg over 12 weeks with a 3-week gap between treatments to cause lung adenocarcinoma in rats. After twenty weeks of EC administration, Janker et al. [22] found that three times of EC injections (1 mg/gm) once a week significantly increased the number of tumor nodules in mice compared to a single injection, even though there was no death or morbidity and a 100% tumor incidence rate.

The amount of time the animals were exposed to EC in the experimental model determines the induction of adenocarcinoma [37]. EC-induced cancer we observed in our model may be due to the active metabolite vinyl carbamate. This potent mutagen encourages the production of electrophilic species that interact with DNA to form 2-oxoethyl adducts like vinyl chloride [38].

The EC+CALE group showed anticancer activity in the current investigation, this result was consistent with Gurukumar et al. [39], who found that the ethanolic extract of *Cassia alata* exhibited good anticancer efficacy against prostate cancer in vivo models using Testosterone and N-methyl N-nitrosourea. In the current study, the prevalence of visible lesions, such as inflammation and RBC extravasation in airway spaces, in the EC+CIS group may be because CIS is harmful to cancer cells and affects normal cells [40].

As a result of the breakdown of urethane or ethyl carbamate, vinyl carbamate and N-hydroxylamine epoxides are produced. These compounds increase the levels of reactive oxygen species (ROS), which causes DNA damage and oxidative stress in the environment of lung cells [41].

The incidence of lung injury has been demonstrated to be significantly influenced by oxidative stress, which interferes with the integrity of proteins and lipids [42]. Moreover, oxidative stress can damage numerous cellular components like DNA, protein, and lipids, which results in carcinogenesis [43].

Repeated exposure to EC significantly altered lipid peroxidation and oxidative stress compared to the CTRL group, as seen by a significant rise in MDA and a decline in GSH in lung homogenate. This could be related to lung cell mitochondrial malfunction and an increase in intrinsic ROS produced in the mitochondria due to the production of electrophilic species [44]. The results for oxidative stress markers in the EC group were consistent with those of Parashar et al. [45], who discovered that EC injections caused lung cancer in rats. Toxic control animals displayed the highest tumor cell counts, lost alveoli, adenocarcinoma structure, elevated SOD levels, and decreased GSH levels after EC administration.

Sagnia et al. [46] demonstrated the free radical scavenging activity of *Cassia alata* methanol plant extract using the DPPH assay, which was consistent with the anti-oxidant characteristics of CALE demonstrated in this work. Flavonoids, phenols, and tannins are present in polar and non-polar *Cassia alata* extracts [47]. Given that they have the ideal underlying chemistry to play the role of free radical scavengers, phenols constitute a significant class of antioxidants [48]. Numerous studies suggested flavonoids could slow tumor cell growth and inhibit tumor cell proliferation [49].

The current study found that EC group rats had significantly lower wild-type EGFR m-RNA expression levels than CTRL rats. This finding was in line with Meseure et al., [50] finding that invasive breast carcinomas had significantly lower levels of wild-type EGFR mRNA and protein expression than normal breast tissues. In contrast to our finding, Janku et al. [51] observed that EGFR overexpression and mutations were also seen in premalignant lung epithelium, squamous cell carcinoma, and metastatic non-small cell lung carcinoma.

Yarden and Pines [52] explained wild-type EGFR low expression due to oncogenic EGFR mutations and major genomic rearrangements in (lung, breast, and ovarian cancers) that typically generate altered receptor endocytosis, which contributes to enhanced signalling characteristics. In cancer, inappropriate EGFR activation is caused by defective receptor endocytosis and trafficking [53]. In the current study, wild-type EGFR mRNA expression was much higher in the EC+CALE group compared to the EC group, attributed to the extract's anti-proliferative characteristics [54].

5. Conclusion

Lung cancer was successfully produced with four intra-peritoneal injections of 1gm/kg/rat ethyl carbamate (EC) once a week for a month with five months latency period. The above results showed the anticancer ability of *Cassia alata* leaves extract in treating chemically-induced lung cancer in male rats through amelioration of lung index, histopathology, oxidative stress biomarkers, and regulation of wild-type EGFR expression in cancerous tissue. Thus, we could conclude that *Cassia alata* leaves extract is used as a new source of anti-cancer natural compounds fighting lung cancer.

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