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Pinus roxburghii and *Nauplius graveolens* Extracts Elevate Apoptotic Gene Markers in C26 Colon Carcinoma Cells Induced in a BALB/c Mouse Model

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Abstract: The present study aimed to evaluate the chemopreventive potential of *Pinus roxburghii* branch (*P. roxburghii*) and *Nauplius graveolens* (*N. graveolens*) extracts against human colorectal cancer (CRC) induced by C26 murine cells in a BALB/c mouse model. Real-time qRT-PCR was used to evaluate the apoptotic pathway by measuring the relative mRNA expression levels of the Bcl-2, Bax, Cas3, NF- κ B, and PI3k genes. At the termination of the 30-day period, blood samples were collected to assay the biomarkers. The results showed a significant increase ($p < 0.05$) in the levels of TGF- β , CEA, CA19-9, malondialdehyde, ALT, AST, ALP, urea, and creatinine in the positive control compared to the negative control group. In addition, the glutathione reductase activity and total antioxidant activity were reduced in the positive control compared to the negative control. The biomarkers mentioned above were restored to almost normal levels after administering a safe dose (1/10) of a lethal dose of *P. roxburghii* and *N. graveolens* extracts. Administration of one-tenth of the LD₅₀ of *P. roxburghii* and *N. graveolens* extracts caused a significant upregulation of the expression of Bax and Cas-3 and downregulation of the Bcl-2, NF- κ B, and PI3k genes vs. the GAPDH gene as a housekeeping gene compared to the control group. Furthermore, the Bax/Bcl-2 ratio increased upon treatment. After administration of *P. roxburghii* and *N. graveolens* at a safe dose (1/10) of a lethal dose, the results showed improvement in both body weight gain and a significant decrease ($p < 0.05$) in tumor volume. Histopathological changes supported these improvements. Conclusively, the research outputs show that *P. roxburghii* and *N. graveolens* extracts can be utilized as potential chemopreventive agents for CRC treatment by stimulating cancer cell apoptosis and suppressing CRC survival and proliferation.

Keywords: *P. roxburghii* branch; *N. graveolens*; LD₅₀; Bax and Cas-3; Bcl-2; NF- κ B; PI3k; TGF- β ; CEA; CA19-9

1. Introduction

Cancer disease is a significant public health concern, as it is considered the second leading cause of mortality annually after cardiovascular death incidences around the

world [1,2]. In 2020, colorectal cancer (CRC) accounted for approximately 10% and 9.4% of cancer incidences and cancer causative deaths worldwide, respectively [3]. Based on global future perspectives, CRC incidences are predicted to exceed 3.2 million by 2040. CRC cases increase due to continuous exposure to hazardous environmental factors attributed to lifestyle shifts and diet-related habits [4]. Currently, the most applied cancer treatments include chemotherapy, radiotherapy, immunotherapy, and surgery. Almost all these treatments involve many deleterious side effects for normal cells together with cancer cells. Consequently, novel treatments with minimal side effects are necessary for cancer control and prevention [5]. More efforts need to be made to explore new alternative anticancer agents that would ultimately slow, reverse, or even prevent cancer cell progression without affecting normal cell function and integrity [6,7].

For ancient decades, herbal remedies derived from naturally grown plants were proven to be highly effective in treating many diseases [8–10]. Despite the continuous increase in herbal therapy popularity around the globe, very little is known about the composition of these remedies' active compounds, and hence, minimal information is known about their mode of action either on a cellular or molecular basis [10,11]. As one of the major categories of herbal remedies, edible plants are considered a rich source of phytochemicals; however, their efficacy in cancer treatment and prevention has not yet been deciphered [12]. Inevitably, investigating plant-based therapeutic agents' chemical and cytotoxic characteristics could shed light on applying some of these plant therapies as novel natural anticancer drugs [13]. Recently, phytochemicals were proven to directly influence cancer cell cycle arrest, proliferation enhancement, and apoptosis initiation [7]. Further studies suggested that the role of phytochemicals is primarily initiated by delaying or even halting the transformation of a healthy cell into undifferentiated, malignant cells [14,15].

Pinus roxburghii (*P. roxburghii*) belongs to the Pinaceae family and is famously known as chir pine and cultivated in El-Orman Botanical Garden, Egypt. *P. roxburghii* is an ancient medicinal plant with multi pharmaceutical and medicinal properties, including anti-inflammatory, analgesic, antidyslipidemic, antioxidant, antibacterial, antifungal, and anticancer activities, respectively [16–19]. Previous studies have demonstrated that *P. roxburghii* has various bioactive constituents such as phenolics, flavonoids, tannins, beta-carotene, and lycopene [20]. Moreover, different polyphenolic compounds isolated from the branch of *P. roxburghii* include catechin, kaempferol, protocatechuic acid, caffeic acid, and gallic acid [21]. *Nauplius graveolens* (Forssk.) Wiklund (*N. graveolens*) (synonyms: *Asteriscus graveolens*, *Bubonium graveolens*, *Odontospermum graveolens*) is one of the species of the Asteraceae family. It is known as Tafss [22], and naturally grows in the inland desert of Wadi Feiran, South Sinai, Egypt. The *N. graveolens* plant displays medicinal and pharmacological characteristics, including antioxidant, anti-inflammatory, antimicrobial, and antitumor activities [23–25]. Phytochemical investigations of the *N. graveolens* extract revealed the diverse distribution of phytoconstituents including polyphenolics, flavonoids, tannins, coumaric, and chlorogenic acid as the major phenolic compounds [23]. Moreover, *N. graveolens* contained a high content of flavonoids and sesquiterpene lactone asteriscunolide isomers; naturally occurring Asteriscunolide A enhances apoptosis in a tumor cell line [26].

Targeting apoptosis could be the main approach in cancer treatment and a strong, active area of research. Apoptosis, one of the main mechanisms controlling cancer cell progression, is mainly triggered by the activation, or silencing of caspases, Bcl2, Bax, NF- κ B, and PI3k genes in response to cell injury, which in turn are responsible for the activation or inactivation of certain cellular substrates, eventually leading to either cell death or survival according to the degree of cell injury [27–29]. Several murine models have been developed to study CRC pathogenesis, preferably reflecting CRC chemoprevention or the most critical chemo-treatment aspects. Selecting the most appropriate murine models will maximize the upscaling of therapies from in vitro laboratory trials to *in vivo* clinical practice. Over the last three decades, colon-26 and adenocarcinoma mouse models (C26 model) have been used for research on the natural history of carcinomas and antitumor therapy to study the

effects of several antitumor agents [30–32]. Although previous studies have classified the major phytoconstituents which may be responsible for the anticancer activity of the two plant extracts, the actual assessment of those compounds was not evaluated *in vivo* in any of these studies. Therefore, the aim of the present study was to assess the *in vivo* anticancer activities of *P. roxburghii* and *N. graveolens* extracts in a BALB/c mouse model.

2. Material and Methods

2.1. Chemicals

Methanol, ethanol, dimethyl sulfoxide (DMSO), and paraformaldehyde (PFA) were obtained from Sigma Chemical Company (St. Louis, MO, USA). RPMI 1640 medium, fetal bovine serum, and trypsin-EDTA were procured from Gibco, Life Technologies Limited (Paisley, UK).

2.2. Extract Preparation

The extraction of *P. roxburghii* and *N. graveolens* plants was carried out by maceration method with 90% aqueous methanol. *P. roxburghii* was grown and obtained from the Ministry of Agriculture, Orman botanical garden, Giza (Authentication number: 2151). After removing the foliar leaves, the branch was cut with scissors into small pieces, which were allowed to dry at 40 °C. Aliquots of 75 g of finely ground flour were extracted with 450 mL of absolute methanol. The methanolic extract was filtered, and the filtrate was concentrated on a Büchi rotary evaporator R-114 (Büchi Labortechnik AG, Flawil, Switzerland). The residue was freeze-dried using a Snijders Freeze Dryer (Tilburg, Holland) and saved in sterile vials at −20 °C. *N. graveolens* was collected from the inland desert of Wadi Feiran, South Sinai, Egypt (Authentication number: 3187). Both plants were identified by the pharmacognosy department, National Research Centre, Giza, Egypt according to taxonomic classification by Boulos (2002) [33]. The whole *N. graveolens* plant was treated similarly to *P. roxburghii* [34]. The extraction yields of *P. roxburghii* and *N. graveolens* plants were 5.2 and 5.5 g/75 g of dry material, respectively.

2.3. Composition of Basal Diet

The basal diet consists of corn starch (46.5%), casein (14%), soybean oil (4%), fiber (5%), mineral mixture (3.5%), and vitamin mixture (1%) as described in AIN-93 M [35].

2.4. Determination of the Median Lethal Dose (LD₅₀)

The median lethal dose (LD₅₀) of *P. roxburghii* and *N. graveolens* extracts was evaluated according to Wilbrandt [36]. Briefly, different *P. roxburghii* and *N. graveolens* extract doses of up to 2000 mg/kg body weight in DMSO were orally administered to male albino mice (six mice per group), and a group of six mice was given the respective amount of DMSO orally and left as a control. The median lethal dose (LD₅₀) of the methanolic extracts of *P. roxburghii* accounted for 1708.3 mg/kg body weight, while that of *N. graveolens* was 1562.5 mg/kg body weight. The 1/10 LD₅₀ of *P. roxburghii* and *N. graveolens* extracts accounted for 170.83 and 156.25 mg/kg body weight, respectively [34].

2.5. Animals

Twenty-four male BALB/c mice weighing 20–25 g were purchased from Schistosoma Biological Supply Centre (SBSC) at Theodore Bilharz Research Institute (TBRI), Imbaba, Giza, Egypt. All the animals were acclimatized for a week under standard husbandry conditions. The animals had been fed a standard pellet diet and water *ad libitum*. Animal handling and experimental procedures were conducted by the Guidelines of Care Laboratory Animals, National Research Centre, and approved by the Research Ethical Committee and the national legislation on lab Animal Rescue and Usage (Ethical Code: 15/097; 4 February 2021).

2.6. Induction of C26 Murine Cells in BALB/c Mice

Murine colon-26 cells were cultured in RPMI 1640 medium supplemented with fetal bovine serum (10%) (Gibco, Life Technologies Limited, Paisley, UK) in a humidified atmosphere (5% CO₂) at 37 °C. Cells were collected using trypsin-EDTA (0.25%) to obtain a single-cell suspension. Cells were counted using a hemocytometer and pelleted via centrifugation at 25 °C (500 × g for 5 min). Mice were injected subcutaneously on the dorsal side with 1 × 10⁶ cells (Figure 1), as described by [37,38].

2.7. Measurement of Body Wasting

Body mass was monitored weekly after inoculation. Tumor size was measured using a Verniercaliper, and tumor volume was measured using the formula $V = (\text{length} \times \text{width}^2)/2$ in mm³ [39,40].

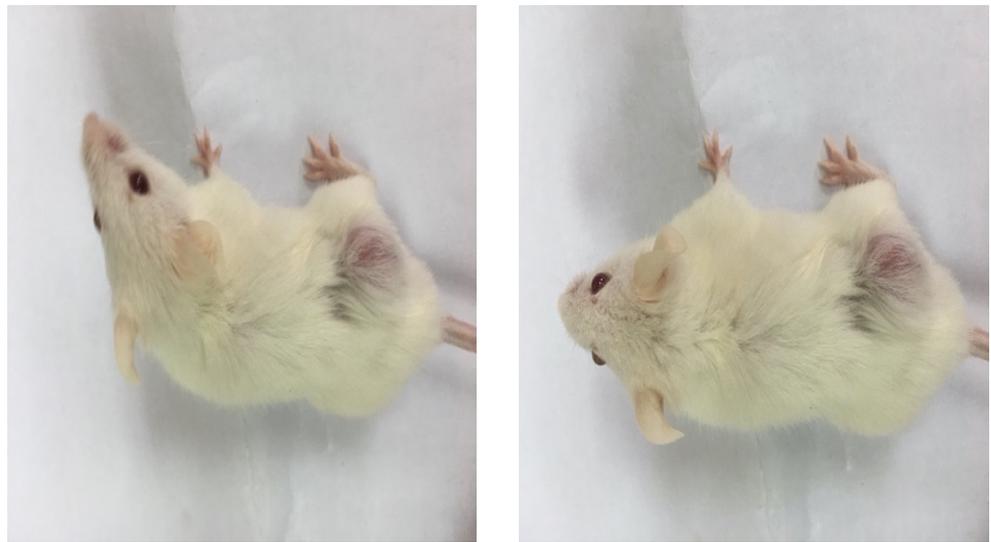


Figure 1. Tumor growth in BALB/c mice.

2.8. Experimental Design

Twenty-four male BALB/c mice were divided into four groups as follows:

Group (1): Negative control received a basal diet.

Group (2): Positive control received a basal diet and was injected intraperitoneally with a respective amount of DMSO.

Group (3): Tumor mice received a basal diet and were injected intraperitoneally with a dose equivalent to 1/10 LD₅₀ of extract of *P. roxburghii* branch day after day.

Group (4): Tumor mice received a basal diet and were injected intraperitoneally with a dose equivalent to 1/10 LD₅₀ of *N. graveolens* extract day after day.

Tumor volumes were measured weekly for four consecutive weeks (one month).

The diet was withdrawn at the end of the one-month feeding trial, and all animals were fasted overnight (12 h). The mice were anesthetized by intramuscular injection with ketamine chloride (24 mg/kg body weight). Blood was sampled from the orbital sinus of the eye and collected in clean, dry test tubes. The serum was separated by centrifugation (1500 × g, 10 min, 4 °C). The serum obtained was used for various biochemical estimations.

2.9. Changes in Body Weight (BW) and Feed Efficiency Ratio (FER)

Body weight and food consumption were recorded by Hsu et al. [41] by the following formula:

$$\text{Changes in body weight} = \text{final body weight} - \text{initial body weight}$$

$$\text{Feed efficiency ratio (FER)} = \text{Body weight gain, g} / \text{Food intake, g}$$

2.10. Biochemical Analyses

Liver function was assessed by measuring liver enzyme activities, such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP), using a kit (Biodiagnostic, Egypt). Serum concentrations of urea and creatinine, the two indicators of kidney function, were determined using kits (Biodiagnostic, Egypt). The assays followed the manufacturer's protocol based on published techniques [42–45]. Glutathione reductase, total antioxidant capacity, and malondialdehyde (MDA) were determined colorimetrically using a kit (Biodiagnostics, Egypt) described by [46–48]. The TGF- β level was assayed using an ELISA kit purchased from Sunlong Biotech Co., Ltd., China. Serum CA 19-9 was assessed using the Cancer Antigen CA 19-9 ELISA Kit (Abcam, Cambridge, UK). Serum carcinoembryonic antigen (CEA) was determined using an ELISA kit (enzyme-linked immunosorbent assay) (Abcam, Cambridge, UK). These assays were carried out based on the manufacturer's instructions.

2.11. RNA Extraction and cDNA Synthesis

RNA was extracted from tissues using the RNeasy mini kit (Qiagen, Hilden, Germany) as designed by the manufacturer. Total RNA purity and concentration were detected by a Nanodrop UV spectrophotometer. cDNA was synthesized using a cDNA synthesis kit (Intron Biotechnology, Seongnam-si, Korea) as stated in the manufacturer's protocol.

2.12. Quantitative Real-Time PCR

Gene expression was tested on the genes Bax, Bcl-2, Cas3, PI3K, and NF- κ B, and GAPDH was used as a housekeeping gene. The specific primers used for quantitative PCR were designed through NCBI BLAST before purchasing from Thermo Fisher Scientific (Waltham, MA, USA) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>, accessed on 1 May 2021), and their sequences are presented in Table 1. A real-time PCR cycler (QIAGEN, Rotor-Gene Q, Valencia, CA) was used to detect the synthesized cDNA copy number. PCR mixtures were set up in a 25 μ L final volume, containing 12.5 μ L SYBR Premix Ex Taq TM (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 6.5 μ L distilled water, 0.5 μ L sense primer (0.2 mM), 0.5 μ L antisense primer (0.2 mM), and 5 μ L of cDNA template. The reaction program was designed with three steps. The first denaturation step was performed at 95.0 $^{\circ}$ C for 3 min. The second step consisted of 40 cycles, each cycle divided into (a) 95.0 $^{\circ}$ C for 15 s; (b) 55.0–60.0 $^{\circ}$ C for 30 s; and (c) 72.0 $^{\circ}$ C for 30 s. The third step consisted of several cycles, which started at 60.0 $^{\circ}$ C and increased approximately 0.5 $^{\circ}$ C every 10 s up to 95.0 $^{\circ}$ C. At the end of each qRT-PCR cycle, a melting curve analysis was designed at 95.0 $^{\circ}$ C, then 55.0–60.0 $^{\circ}$ C, followed by 95.0 $^{\circ}$ C to check the primer quality. Each sample was prepared in triplicate, and the variability of the samples was evaluated using geometric standard deviations. The geometric mean of the triplicate run for each gene was normalized to the GAPDH geometric mean.

Table 1. Primer sequences used for qRT-PCR.

Primers	Sequences
Bcl-2	F: 5'TGGGATGCCTTTGTGGAAC 3' R: 5'CATATTTGTTTGGGGCAGGTC3'
Bax	F: 5'TGCTACAGGGTTTCATCCAG3' R: 5'ATCCACATCAGCAATCATCC3'
Cas-3	F: 5'GCTGGACTGCGGTATTGAGA3' R: 5'CCATGACCCGTCCTTGA3'
NF-kB	F: 5'GCAAAGGGAACATTCCGATAT3' R: 5'GCGACATCACATGGAATCTA3'
PI3k	F: 5'GTGTCAGCGCTCTCCGCC3' R: 5'CTGATAATTGATGTAGG3'
GAPDH	F: 5'CATTCAAGACCGGACAGAGG3' R: 5'ACATACTCAGCACCAGCATCACC3'

2.13. Histopathological Examination

Tumors were separated and fixed in 4% paraformaldehyde (PFA) for 24 h. The tissues were washed with tap water, followed by sequential soaking in diluted methanol, ethanol, and absolute ethyl alcohol for dehydration. The specimens were cleared in xylene, embedded in paraffin, and left in a hot air oven at 56 °C for 24 h. Paraffin bee wax tissue blocks were prepared for sectioning at 4 microns at room temperature by a sledge microtome (SLEE medical, Nieder-Olm, Germany). The final tissue sections were embedded on glass slides, deparaffinized, and stained with hematoxylin and eosin for examination by a light microscope [49].

2.14. Statistical Analyses

Statistical analysis was implemented using GraphPad Prism software 8.0 (San Diego, CA, USA). The results are expressed as the mean ± standard error of the mean (SEM) of three independent experiments. Data are presented as the means ± SDs and estimated by one-way analysis of variance (ANOVA) performed using SPSS 19.0 (SPSS Ltd., Surrey, UK). Duncan’s multiple range test (DMRT) was used to detect individual comparisons. Differences were considered significant at $p < 0.05$.

3. Results and Discussion

The data in Figure 2 illustrate the effect of *P. roxburghii* and *N. graveolens* extracts on the tumor growth of mice. The colon tumor volume increased in the positive control during the baseline scan and the first week. In the positive control, tumor volume increased significantly in the second, third, and fourth weeks. Tumor volume can be considered a critical prognostic factor for assessing several types of cancers [50]. Tullie et al. [51] reported a significant correlation between tumor volume and survival rate. Huang et al. [52] explained that large-volume tumors were proportionally correlated with increased cancer risk at both the cellular and molecular levels. Furthermore, data reporting that tumor volume was a potent independent factor revealed the prognosis of several types of cancers, such as lung, nasopharyngeal, neck, head, and other malignant tumors [53,54]. Administration of one-tenth of the LD₅₀ of *P. roxburghii* and *N. graveolens* extracts showed a significant decrease in tumor volume every subsequent week.

The data in Table 2 represent the effect of *P. roxburghii* and *N. graveolens* extracts on the nutritional status of mice. During the development of cancer in the BALB/C mouse model, there was a significant decrease in body weight gain, food intake, and the food efficiency ratio in the positive control (19.36, 177.54, and 0.10, respectively) compared to the negative control (27.11, 189.21, and 0.14, respectively). Generally, body weight decrease is a common side effect in CRC patients. Consequently, research studies have reported that weight control is an essential criterion in CRC survival [55]. Weight loss was significantly linked to increasing colorectal cancer and mortality [56]. However, Diculescu et al. [57], stated that the weight loss index is an independent prediagnostic factor for colorectal cancer progression. Barber et al. [58] also found that body weight loss caused by cancer may be due to anorexia, substrate metabolism deficiencies, and hypermetabolism. Administration of 1/10 LD₅₀ of *P. roxburghii* and *N. graveolens* extracts showed improvement in body weight gain, food intake, and feed efficiency ratio by 24.52, 25.23, 184.37, 186.29, 0.13, and 0.13, respectively.

Table 2. Nutritional parameters of different groups.

Parameters Groups	Body Weight Gain (BWG) (g)	Food Intake (FI) (g)	Feed Efficiency Ratio (FER)
Negative control	27.11 ± 0.37 ^a	189.21 ± 0.53 ^a	0.14
Positive control	19.36 ± 0.64 ^c	177.54 ± 0.66 ^c	0.10
<i>P. roxburghii</i>	24.52 ± 0.58 ^b	184.37 ± 0.71 ^b	0.13
<i>N. graveolens</i>	25.23 ± 0.45 ^b	186.29 ± 0.49 ^b	0.13

All values are represented as the mean ± S.E. Means with different letters are significantly different ($p < 0.05$).

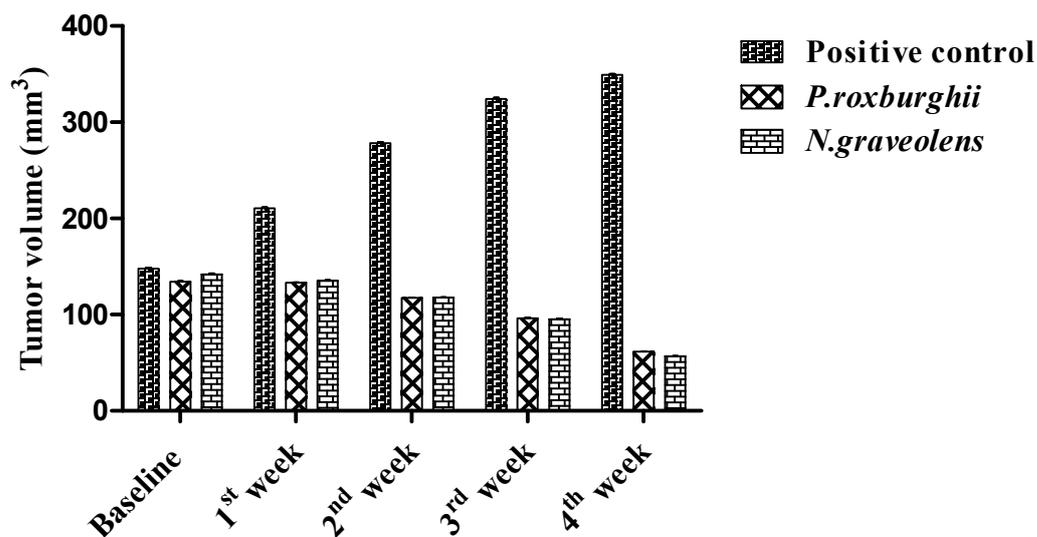


Figure 2. Average tumor volume per week in different groups.

The data presented in Figure 3 show the results of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) activities in the studied groups. The data exhibited a significant increase in serum ALT, AST, and ALP activities in the positive control compared to the negative control. The activities of ALT, AST, and ALP were significantly decreased after administration of 1/10 LD₅₀ of *P. roxburghii* and *N. graveolens* extracts. The conversion of normal cells to cancer cells and cancer cell proliferation lead to abnormal serum enzyme synthesis [59]. ALT, AST, and ALP are major indicator enzymes for liver diagnosis and management [60]. ALT and AST are found inside hepatocytes and are released when the liver is damaged. Liver infection is usually associated with mitochondrial damage, which leads to the immediate release of ALT and AST into the blood circulation and eventually a dramatic increase in these enzyme levels [61]. ALT and AST have a vital role in the prognosis of some cancer types [62,63].

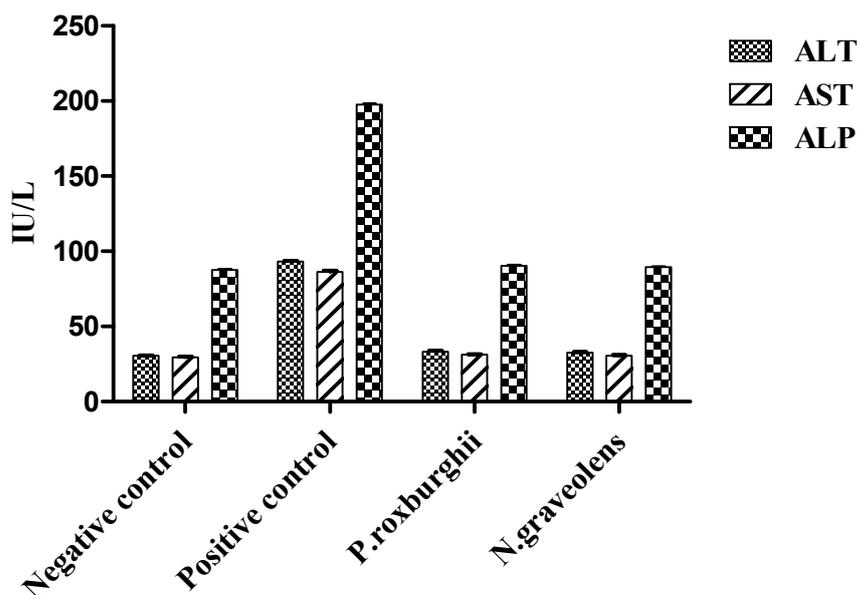


Figure 3. Serum ALT, AST, and ALP activities in different groups.

Serum ALT, AST, and ALP activities were significantly increased in the positive control, representing the degree of severity of hepatic tissue injury reported by Ahn et al. [64]. The alteration of hepatocyte plasma membrane permeability in hepatocyte cancer cells leads

to leakage of cellular enzymes. This ultimately increases the concentrations of these markers in the serum and decreases them in hepatocytes. The activities of ALT and AST were significantly lowered after administration of 1/10 LD₅₀ of *P. roxburghii* and *N. graveolens* extracts. Alkaline phosphatase (ALP) is an enzyme that has been proven to be involved in the transport of metabolites across cellular membranes, proteins and certain enzyme synthesis, glycogen metabolism, and other secretory activities [65]. ALP activity measurement is used as a tumor marker in the early detection and diagnosis of cancer [66]. Lakshmi and Subramanian [67] observed that an elevated level of ALP activity was detected in cancer-bearing animals because of a disturbance in the secretory activity or transport of metabolites of cancer cells. The activity of ALP was significantly decreased after administration of 1/10 LD₅₀ of *P. roxburghii* and *N. graveolens* extracts.

The data in Figure 4 show the serum urea and creatinine levels in the different groups. Serum urea and creatinine levels increased significantly in the positive control group compared to the negative control group. Meanwhile, the administration of 1/10 LD₅₀ of *P. roxburghii* and *N. graveolens* extracts significantly decreased serum urea and creatinine levels. Impaired kidney function is one of the most detectable characteristics in cancer patients. Elevated urea and creatinine levels in the bloodstream of cancer patients, which eventually occur due to renal cell degeneration, are vital in cancer disease diagnosis and management [68–70]. The results showed that blood serum creatinine and urea levels were significantly increased in colon cancer-induced mouse models. The results corresponded with those of Raj et al. [71], who reported a detectable increase in serum creatinine levels in breast cancer-induced rats. Increased creatinine levels were also detected in the plasma of cervical cancer patients, as reported by Koji et al. [72], and were considered the major risk factor for cervical cancer. In contrast, a significant reduction in serum creatinine and urea levels was detected after administration of 1/10 LD₅₀ of *P. roxburghii* and *N. graveolens* extracts. These findings corresponded with the results of Falconer et al. [73]. They observed a significant increase in the creatinine level in all cancer types compared to the normal subjects.

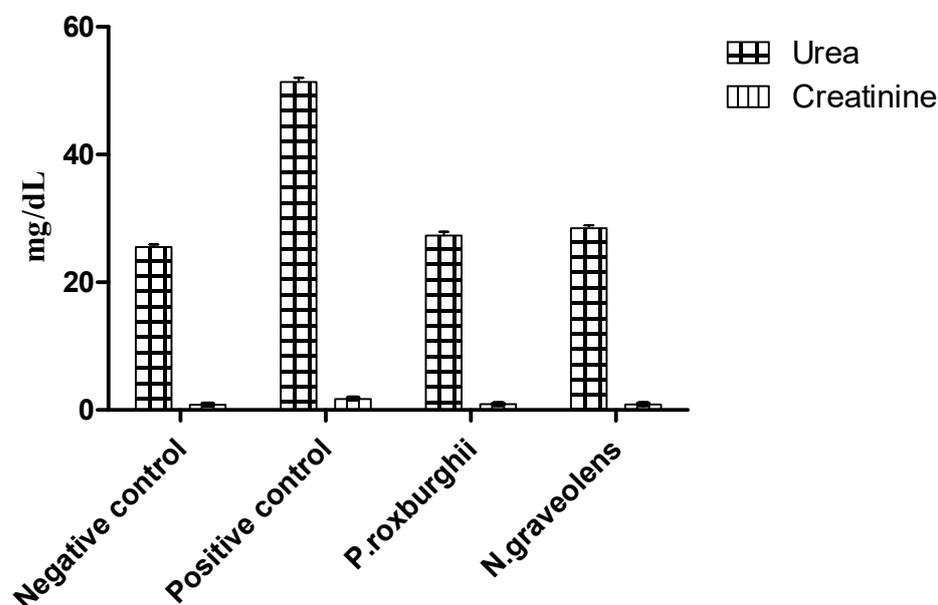


Figure 4. Levels of serum urea and creatinine in different groups.

The data presented in Figures 5–7 illustrate the activity of glutathione reductase, total antioxidant capacity, and malondialdehyde levels. The data showed a significant decrease in glutathione reductase activity in the positive control compared to the negative control. Administration of 1/10 LD₅₀ of *P. roxburghii* and *N. graveolens* extracts significantly increases serum GR activity. Moreover, the total antioxidant level decreased significantly

in the positive control compared to the negative control. Administration of 1/10LD₅₀ of *P. roxburghii* and *N. graveolens* extracts showed a significant increase in serum TAC levels. In contrast, a significant increase was observed in serum malondialdehyde levels in the positive control compared to the negative control. Administration of 1/10 LD₅₀ of *P. roxburghii* and *N. graveolens* extracts significantly decreased serum MDA levels. Antioxidant activity can be estimated in humans, which reflects the disturbance in redox equilibrium in body fluids, tissues, or organs under different pathological conditions, including tumor development [74]. Generally, oxidative stress and inflammation are associated with substrate metabolic disturbances because of an environmental effect that eventually leads to the development of CRC [75]. The results showed lower TAC levels in the positive control group than in the negative control group, indicating a weakened antioxidant barrier resulting from overproduction of free radicals. Administration of 1/10 LD₅₀ of *P. roxburghii* and *N. graveolens* extracts showed a significant increase in the TAC level.

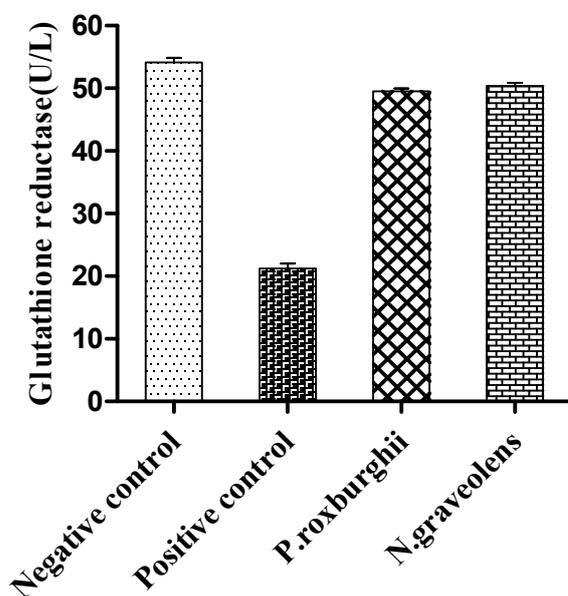


Figure 5. Serum glutathione reductase activity in different groups.

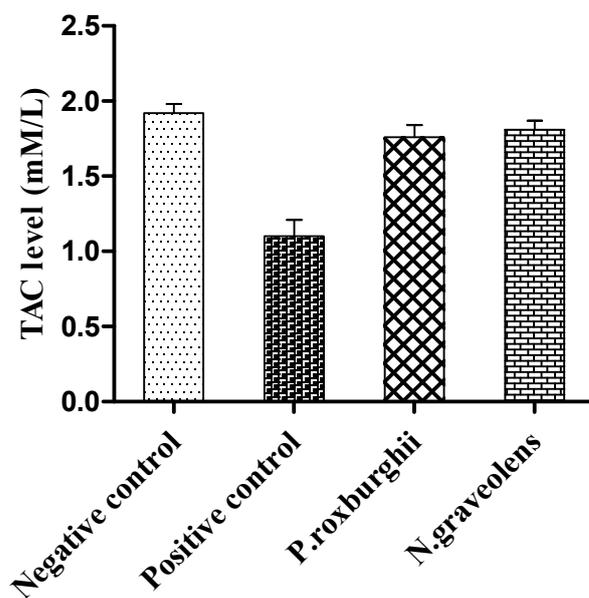


Figure 6. Level of serum total antioxidant capacity in different groups.

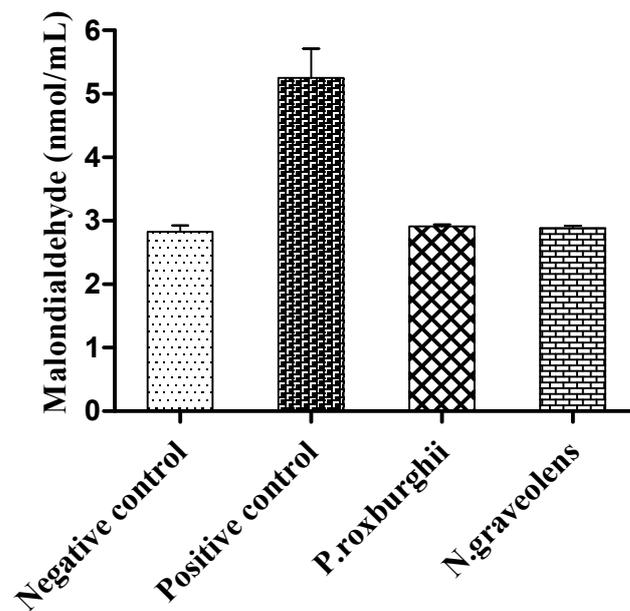


Figure 7. Level of serum malondialdehyde in different groups.

The process of colorectal cancer initiation and progression involves the interaction of various physiological factors, including the overproduction of reactive oxygen species (ROS) and malondialdehyde (MDA) formation. ROS are excessively produced in gastrointestinal tract chronic infection because of lipid peroxidation that eventually leads to cellular protein damage and carcinogenesis induction [76,77]. Malondialdehyde (MDA) (final product of the lipoperoxidation process) is a strong electrophilic compound that interacts with cell nucleophiles to form MDA oligomers that can be used for cancer detection and as a prediagnostic index for CRC patients [78]. MDA is a major molecule responsible for the highest mutagenicity and carcinogenicity [79]. In the present study, the MDA level was significantly increased in the positive control compared to the negative control group. Administration of 1/10 LD₅₀ of *P. roxburghii* and *N. graveolens* extracts showed a significant decrease in the MDA level. Glutathione reductase (GR) is the most important cellular antioxidant enzyme. In its reduced form, GR can capture reactive oxygen and nitrogen species and then participate in the control of redox homeostasis. GR usually accumulates in cellular regions of high electron flux, where reactive species are generated. Mayo et al. [80] reported that ordinary free radicals, such as hydroxyl, alkoxy, superoxide anion, and peroxy radicals, may alter the GR structures, resulting in the inactivation of the enzyme and eventually lowering its activity. In CRC cases, GR content might be exhausted, which subjects the patients to redox imbalance and oxidative damage [81]. The level of GR activity was significantly decreased in the positive control compared to the control group. Administration of 1/10 LD₅₀ of *P. roxburghii* and *N. graveolens* extracts significantly increased GR activity.

The data presented in Figures 8–10 illustrate the effect of *P. roxburghii* and *N. graveolens* extracts on transforming growth factor-beta (TGF- β) and serum carcinoembryonic antigen (CEA) and cancer antigen (CA19-9) levels in different groups. The results revealed a significant elevation in serum TGF- β levels in the positive control compared to the negative control. Administration of 1/10 LD₅₀ of *P. roxburghii* and *N. graveolens* extracts showed a significant decrease in serum TGF- β levels. Furthermore, a significant increase was observed in serum CEA levels in the positive control compared to the negative control. Administration of one-tenth of the LD₅₀ of *P. roxburghii* and *N. graveolens* extracts showed a significant decrease in serum CEA levels. The data showed a significant increase in the level of CA19-9 in the positive control compared to the negative control. Administration of one-tenth of the LD₅₀ of *P. roxburghii* and *N. graveolens* extracts significantly lowered serum CA19-9 levels.

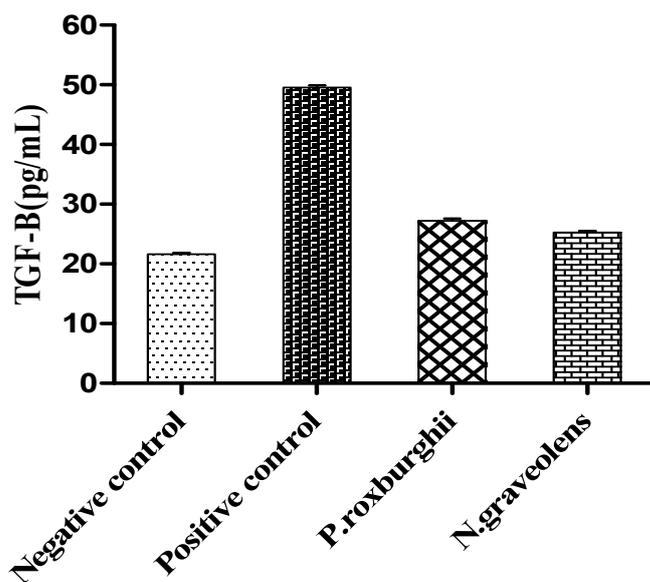


Figure 8. Level of serum TGF-β in different groups.

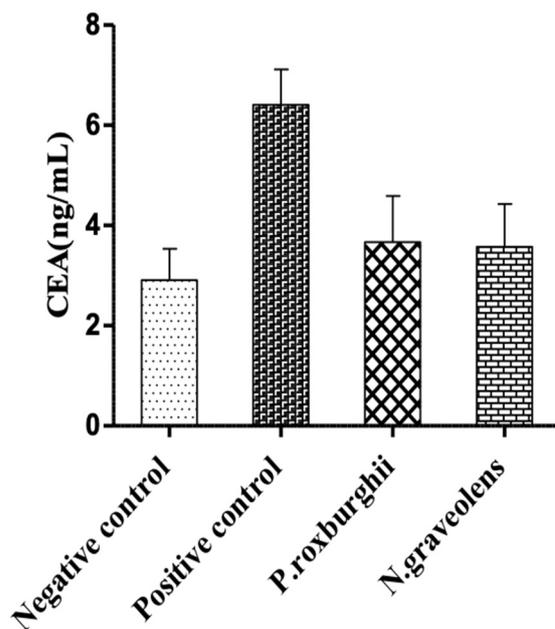


Figure 9. Level of serum CEA in different groups.

According to Jung et al. [82], transforming growth factor-beta (TGF-β) is a signaling pathway for controlling cell growth, differentiation, multiplication, homeostasis, and apoptosis. Although TGF-β is responsible for signaling the arrest of epithelial cell growth in normal tissues, it enhances tumor cell development in tissues with cancer progression [83]. Carcinoembryonic antigen (CEA) is a membrane-bound glycoprotein expressed by cancer and, to some level, by normal epithelial cells of the gastrointestinal tract. CEA is currently recognized as a tumor biomarker for the analytical detection and diagnosis of colon cancer (CC) [84]. An increased blood level of CEA indicates metastasis and cancer prognosis [85]. CEA is implicated in various biological processes of neoplasia, such as cell adhesion, metastasis, suppression of cellular immune mechanisms, and inhibition of apoptosis [86]. Cancer antigen 19-9 (CA19-9) is used as a tumor marker and plays a vital role in the process of tumor progression [87]. It has also been detected as a prognostic biomarker for colorectal cancer [88,89]. Combining three tumor markers, TGF-β, CEA, and CA19-9, provides a more sensitive method for colorectal cancer detection and prognosis. The results of the current

study revealed that there was a significant increase in serum TGF- β , CEA, and CA19.9 levels in the positive control compared to the negative control group. Administration of 1/10 LD₅₀ of *P. roxburghii* and *N. graveolens* extracts decreased serum TGF- β , CEA, and CA19.9 levels.

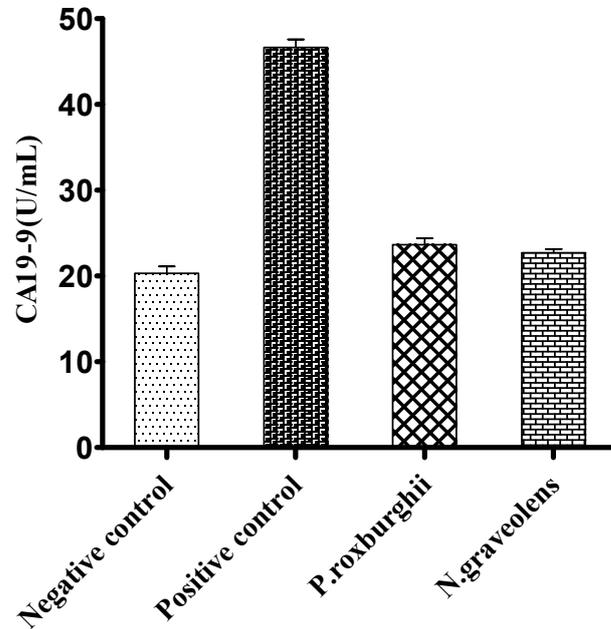


Figure 10. Level of serum CA19-9 in different groups.

The results of the expression of the Bcl-2, Bax, Cas3, NF- κ B, and PI3k genes in the different groups are shown in Figure 11. Real-time qRT-PCR was used to evaluate the expression of several apoptotic genes, including Bcl-2, Bax, cleaved Cas3, NF- κ B, and PI3k. Administration of one-tenth of the LD₅₀ of *P. roxburghii* and *N. graveolens* extracts caused a significant upregulation of the expression of the Bax gene and downregulation of the Bcl-2 gene. The Bax/Bcl-2 ratio was increased upon treatment after administration of one-tenth of the LD₅₀ of *P. roxburghii* and *N. graveolens*, with ratios of 2.01 and 1.91, respectively. In addition, the Cas-3 gene was upregulated after administration of one-tenth of the LD₅₀ of *P. roxburghii* and *N. graveolens* extracts. Meanwhile, administration of one-tenth of the LD₅₀ of *P. roxburghii* and *N. graveolens* extracts activated apoptosis by downregulating the expression of the NF- κ B and PI3K genes.

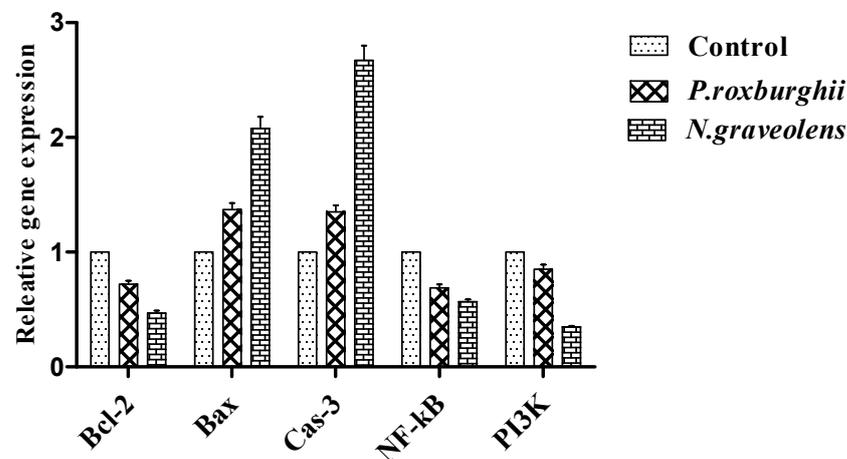


Figure 11. Effect of *P. roxburghii* and *N. graveolens* extracts on Bcl-2, Bax, Cas3, NF- κ B, and PI3k genes relative to the GAPDH gene in BALB/c mice.

Previous studies demonstrated that these apoptotic proteins are crucial in tumor suppression, arrest, and finally, execution of cancer cells [90,91]. Phenolic compounds may play a vital role in the down- and upregulation of the selected genes under study.

Phytochemical analysis of both *P. roxburghii* and *N. graveolens* extracts showed that they are highly rich in catechin, chlorogenic acid, and gallic acid. Other polyphenol compounds, such as p-coumaric, syringic, caffeic, and p-hydroxybenzoic acids, were detected in both extracts. Cinnamic acid, rosmarinic acid, and apigenin-7-glucoside were undetected in the *P. roxburghii* extract, which was rich in other compounds, such as ferulic and protocatechuic acids [20,21,23]. Similarly, Granado-Serrano et al. [92], found that epicatechin induced NF- κ B, PI3K/AKT, and ERK signaling in HepG2 cells. Moradzadeh et al. [93] reported that epicatechin increased the Bax/Bcl-2 ratio, downregulated PI3K, Akt, and Bcl-2, and upregulated p53, p21, caspase-3, and caspase-9 in a human breast cancer cell line (T47D). Gallic acid suppresses cancer cell proliferation and triggers apoptosis in a dual process through the downregulation of Bcl-2 and upregulation of Bax [94]. Sajid et al. [95], observed that the essential oil of *P. roxburghii* inhibited the expression of NF- κ B-regulated gene products implicated in cell survival (Bcl-2, Bcl-xL, c-Myc) and MMP-9. Intriguingly, in the current study, the chlorogenic acid and rutin found in the *N. graveolens* extract may exhibit antitumor properties similar to the results obtained by [96,97]. Moreover, *N. graveolens* has a good source of apigenin-7-glucoside; apigenin intake blocks the phosphorylation and further degradation of nuclear factor of kappa light polypeptide gene stimulator in the B-cell inhibitor alpha (I κ B α) by the inhibitor of NF- κ B kinase (IKK) activation, which in turn leads to the inhibition of NF- κ B activation. Apigenin intake downregulated Bcl-2 and Bcl-xL and suppressed NF- κ B activation [98]. Rosmarinic acid intake downregulated the expression of Bcl-2 and elevated Bax in human colon adenocarcinoma (HT-29) [99]. Li et al. [100] found that rosmarinic acid downregulated the expression of Bcl-2 and upregulated the expression of Bax in breast cancer stem-like cancer (BCSCs). The other chemical compounds were detected in *P. roxburghii* and *N. graveolens* extracts, including p-hydroxybenzoic acid, caffeic, syringic, vanillic, p-coumaric, and kaempferol. Caffeic acid supplementation led to cell cycle modulation, suppression of colony formation, alterations in the expression of caspases, and induction of apoptosis [101]. Abaza et al. [102] found that syringic acid has antimitogenic and chemosensitizing activities against human colorectal cancer, thus halting the cell cycle and inducing apoptosis. Vanillic acid suppressed angiogenesis and proliferation which led to cell cycle arrest in HCT-116 cells [103]. Moreover, supplementation with p-coumaric acid induced apoptosis through modulation of the Bax/Bcl-2 ratio and improvement in detoxification [104].

Histopathological Observations of Tissues

The histopathological examination of muscle fiber sections from the control group revealed normal muscle fiber (black arrow) and entangling fibrous tissue (red arrow) (HE, $\times 400$) (Figure 12). Positive control sections showed malignant growth formed of sheets of malignant epithelial cells with large nuclei and an increased the nucleocytoplasmic ratio (black arrow), showing moderate anaplasia (Figure 13). Sections of mice administered one-tenth the LD₅₀ of *P. roxburghii* extract are illustrated in Figure 14 and showed a scattered sheet of dysplastic epithelial cells with rounded nuclei with an increased nucleocytoplasmic ratio (black arrow), with many apoptotic cells and necrosis (red arrows).

Sections of mice administered one-tenth the LD₅₀ of *N. graveolens* extract are illustrated in Figure 15 and showed a scattered sheet of epithelial cells with rounded nuclei with an increased nucleocytoplasmic ratio (black arrow), with many degenerated/necrotic and/or apoptotic cells (red arrows).

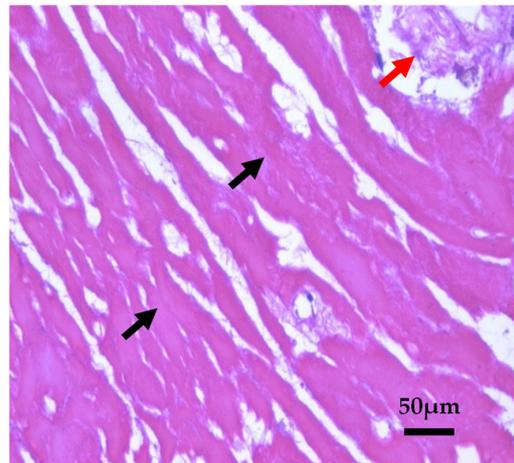


Figure 12. Sections of muscle fibers from the control group showed normal muscle fibers (**black arrow**) and entangling fibrous tissue (**red arrow**) (HE, $\times 400$).

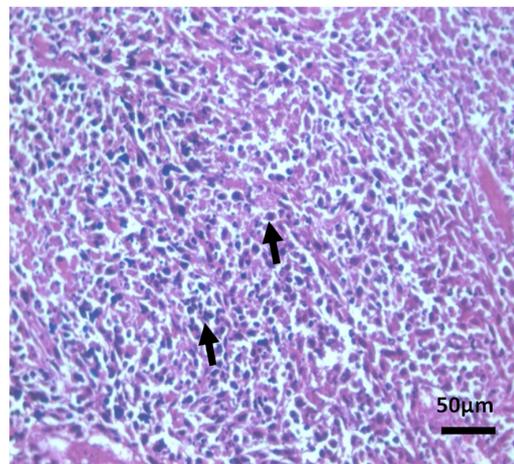


Figure 13. Sections of the positive control group showed malignant growth formed of sheets of malignant epithelial cells with large nuclei with an increased nucleocytoplasmic ratio (**black arrow**), showing a moderate degree of anaplasia (HE, $\times 400$).

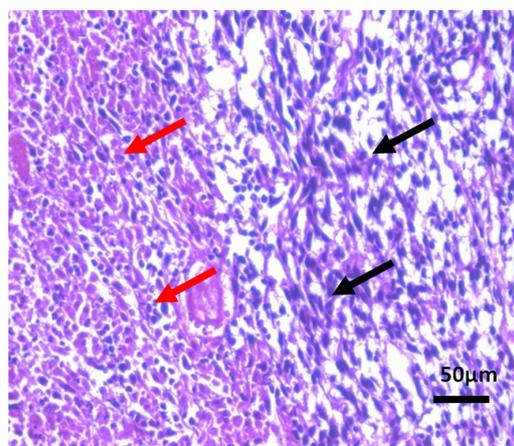


Figure 14. Sections treated with *P. roxburghii* extract showed a scattered sheet of dysplastic epithelial cells with rounded nuclei with an increased nucleocytoplasmic ratio (**black arrow**), with many apoptotic cells and necrosis (**red arrows**) (HE, $\times 400$).

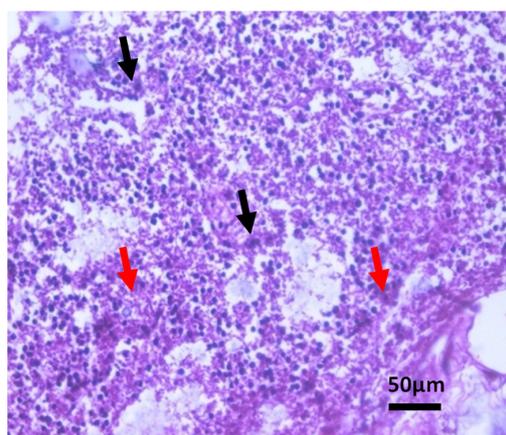


Figure 15. Sections treated with *N. graveolens* extract showed a scattered sheet of epithelial cells with rounded nuclei with an increased nucleocytoplasmic ratio (**black arrow**), with many degenerated/necrotic and/or apoptotic cells (**red arrows**) (HE, $\times 400$).

4. Conclusions

The research outputs of the current study concluded that administration of one-tenth of the LD_{50} of *P. roxburghii* and *N. graveolens* extracts possess anti-proliferative activity by suppressing the tumor growth rate in the BALB/c mouse model. General enhancement in liver and kidney functions, antioxidant activity, and reduction in tumor markers blood levels were detected in the BALB/c mouse model after the administration of the selected dose. Furthermore, the apoptotic cell cycle was enhanced by optimizing the regulation of various apoptotic gene marker expression levels. These improvements were supported by histopathological changes. However, further clinical trials are needed to be performed to confirm these extracts as a potential anticancer drug.

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