**Annona squamosa** L. Extract-Loaded Niosome and Its Anti-Ehrlich Ascites’ Carcinoma Activity

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**ABSTRACT:** Current research is focused on cancer treatments other than chemotherapy medications, particularly those derived from natural sources. The goal of this work was to look at the anticancer and biomarker properties of a methanolic extract of *Annona squamosa* leaves and their extract-loaded noisome. *A. squamosa* leaves extract and their leaves extract-loaded noisome were prepared. Transmission electron microscopy was used to screen the size of the niosomes loaded with the *A. squamosa* L. leaves extract. The tumor size, blood picture (hemoglobin, red blood cells, white blood cells), liver functions, kidney function, oxidative stress, and inflammatory markers were evaluated to assess the potential anticancer activity of the *A. squamosa* leaves extract and *A. squamosa* leaves extract-loaded noisome in Ehrlich ascites carcinoma. *A. squamosa* L. leaves extract was found to be an effective anticancer treatment. The protective effect of the loaded extract showed more significant results. All treated groups showed a lower tumor volume compared to the positive control. Liver and kidney functions were improved, and inflammatory markers were decreased. Oxidative stress was improved in tumor, liver, and kidney tissues. *A. squamosa* leaves contain major anticancer compounds that in general help most enzymes of the liver and kidney and other injured organs to return to their normal levels.

**INTRODUCTION**

Cancer is a significant global public health issue and has the second highest mortality rate in the United States, accounting for about 610,000 deaths in 2022.¹ The use of natural plant elements as an alternative to chemotherapy or chemopreventive drugs is an effective strategy in the fight against cancer, especially with the possibility of targeting cancerous cells without healthy cells.²

*Annona squamosa* is a midsize tree belonging to the **Annonaceae** family. In many places of the world, it grows abundantly: for example, in Africa (Egypt), Asia, Australia, and America. It is known by its various names, such as sugar apple and sweetsop in English, Sirafal in Hindi, and Matomoko in Kenya.³ *A. squamosa’s* anticancer activity is selective for distinct cancer cells, as it has antiproliferative⁴ and antioxidant activity.³ Phytochemicals obtained from the plant, such as terpenoids, flavonoids, alkaloids, glycosides, and phenols, show subtle effects on body physiology and have the desired effects. They have an antioxidant ability that quenches the free radicals and therefore they act as cancer chemo-preventive agents. In comparison to synthetic chemotherapeutic agents, plant-based medicines are found to be safer due to their less-toxic side effects.⁵

Nanoparticles account for nanoscale applications, providing a high surface area and efficient interaction, and working as carriers for molecules.⁶ Nanoparticles can be selectively used to target the delivery of therapeutic drugs⁷ and decrease drug resistance by cancer cells.⁸ Niosomes are distinctive drug nanocarriers developed by non-ionic surfactants and cholesterol self-association in an aqueous solution.¹⁰ They can encapsulate hydrophilic and hydrophobic drugs with adequate efficiency. Niosomes are biocompatible, low-cost, biodegradable, and stable.¹¹,¹²

Previous studies have tried to encapsulate the *A. squamosa* extract for cancer treatment. Fadholly et al., 2019¹³ prepared chitosan nanoparticles-loaded *A. squamosa* leaves extract against human colon cancer (WiDr) cell lines. They showed potent cytotoxicity, increasing caspase-3 expression significantly and cell cycle arrest in the G2/M phase. According to...
the free A. squamosa leaf extract increased the expression of proliferative and apoptotic markers, which in turn enhanced the extract’s anticancer effect on breast cancer cell lines. Additionally, they conducted an in vivo investigation, and it was found that the treatment of rats with the extracts had an impact on the tumor size, proliferative, and apoptotic indicators.

The current study differs from earlier ones in that it first prepares an extract of A. squamosa leaves, loads it onto niosomes with active ingredients, evaluates the success of the loading and release of the extract, and then evaluates the effectiveness on tumor-bearing mice. This study investigated the antitumoral activity and biomarker properties of the methyl extract of A. squamosa leaves in the Ehrlich ascites carcinoma (EAC) model. Efficiency comparison between the A. squamosa leaves extract (ASLE) and A. squamosa leaves extract-loaded noisome (ASLELN) was done by determining the liver and kidney functions, inflammatory markers, blood picture, oxidative stress, and tumor size.

**RESULTS**

**Loading Efficiency.** In the current work, niosomes exhibit a high loading efficiency for the ASL extract (93 ± 3%).

**UV Absorption Spectrum.** The ultraviolet (UV) absorption spectrum for the ASLE shows an absorption band at wavelength 200–300 nm, one at 400–500 nm, and a small peak at 700 nm, while the loaded niosomes show an enhancement for the peak at 700 nm and the band at 400–500 nm with a slight shift in the position due to the presence of the extract loaded in the niosome membrane (Figure 1).

**Transmission Electron Microscopy.** Under a zooming of 200 nm, the transmission electron microscopy (TEM) image presented the indication that the nanoparticles were highly monodispersed without aggregation. The prepared nanoparticles were well isolated in a spherical shape with an average nanoparticle diameter of 220 nm (Figure 2).

**In Vitro Extract Release.** The burst discharge of free ASLE from the dialysis bag took 5 h to attain equilibrium. In the case of the ASLE loaded into the niosomes, the nano loading, besides its ability to save the extract from expected biodegradation in the body, promoted the sustained release of ASLE over 10 h (Figure 3). Niosomes are stable carriers for ASLE, and the slow release reinforces the therapeutic effect of the extract.

**Effect of Tested Extracts on the Ehrlich Tumor Size.** Figure 4 shows the effect of ASLE and ASLELN on the
The development of Ehrlich’s volume compared to the Ehrlich solid tumor group (EST) not treated with any formulation. A significant decrease in tumor volume was detected in group 4, which was administered ASLELN before EST formation (Nano form + EST) by 59% ($0.132 \pm 0.011$), and group 6, which was administered ASLELN after EST formation (EST + Nano form) by 63% ($0.120 \pm 0.008$), compared to the EST control group ($0.325 \pm 0.016$).

**Hematological Parameters.** The Ehrlich solid tumor (EST) group shows a considerable significant drop in Hb %, lymphocyte, and red blood cell (RBC) count by 29% (10.87 ± 0.06), 22% (53.00 ± 1.73), and 36% (3.30 ± 0.17), respectively, as compared with the normal group (14.90 ± 0.30, 68.17 ± 1.01, and 5.14 ± 0.07, respectively), accompanied by a significant increase in white blood cell (WBC) count and percentage of neutrophils and monocytes by 71% (10.87 ± 0.06), 31% (30.83 ± 0.41), and 144% (2.16 ± 0.14), respectively, as compared with the EST group, accompanied by a significant decrease in WBC count and percentage of neutrophils and monocytes by 39% (6.60 ± 0.05), 15% (26.33 ± 0.72), and 35% (1.41 ± 0.06), respectively, and by 22% (8.50 ± 0.34), 27% (22.37 ± 0.49), and 36% (4.49 ± 0.14), respectively, as compared with the EST group.

| groups          | Hb content (g%) | RBC ($\times 10^6$ (mm$^3$)) | WBC ($\times 10^6$ (mm$^3$)) | lymphocytes (%) | neutrophils (%) | monocytes (%) |
|-----------------|----------------|-----------------------------|-----------------------------|-----------------|----------------|--------------|
| normal          | 14.40 ± 0.30   | 5.14 ± 0.07                 | 6.36 ± 0.18                 | 68.17 ± 1.01    | 23.50 ± 0.86   | 0.89 ± 0.01   |
| EST             | 10.53b ± 0.29  | 3.30b ± 0.17                | 10.87b ± 0.06               | 53.00b ± 1.73   | 30.83b ± 0.41  | 2.16b ± 0.14  |
| ASLE pre-EST    | 12.20b ± 0.15  | 4.08b ± 0.08                 | 8.50b ± 0.28                | 59.67b ± 0.33   | 29.08b ± 1.08  | 1.80b ± 0.11  |
| ASLELN pre-EST  | 13.47b ± 0.29  | 4.74b ± 0.17                | 6.60 ± 0.05                 | 64.53b ± 0.90   | 26.33b ± 0.72  | 1.41b ± 0.06  |
| EST with ASLE   | 12.33b ± 0.16  | 4.21b ± 0.11                 | 8.50b ± 0.28                | 66.67 ± 1.33    | 28.90 ± 0.49   | 0.96b ± 0.03  |
| EST with ASLELN | 12.67b ± 0.16  | 4.49b ± 0.14                 | 8.50b ± 0.34                | 68.37 ± 1.38    | 22.37 ± 1.38   | 0.94b ± 0.03  |

*Each value indicates the mean ± SEM. bSignificantly different from the normal group at $p < 0.05$. cSignificantly different from the control EST group at $p < 0.05$. Statistical analysis was performed by one-way ANOVA followed by Tukey multiple comparison test.

**Figure 5.** Levels of inflammatory markers (a) TNF-α, (b) NF-kB, and (c) COX-2 in the serum of different studied groups. Each value indicates the mean ± SEM. *Significantly different from the normal group at $p < 0.05$. #Significantly different from the control EST group at $p < 0.05$. Statistical analysis was performed by one-way ANOVA followed by Tukey multiple comparison test.
1.38), and 55% (0.96 ± 0.03), respectively, as compared with the EST group (P < 0.05, Table 1).

**Inflammatory Markers in Serum.** Figure 5 shows a significant increase in the serum levels of COX-2, TNF-α, and NF-κB in the EST group by 4-fold (54.50 ± 23.40), 4.8-fold (31.80 ± 15.46), and 4.6-fold (3.16 ± 0.20), respectively, as compared to the normal group (132.00 ± 2.88, 65.87 ± 4.14, 0.69 ± 0.05). On the contrary, pre-administration of ASLELN in the EST inoculation group (Gp 4) and post-administration of ASLELN in the EST inoculation group (Gp 6) showed a significant decrease in the serum levels of COX-2, TNF-α, and NF-κB by 72% (151.70 ± 8.51), 72% (87.80 ± 3.15), respectively, and 72% (0.88 ± 0.07), and by 60% (217.00 ± 15.59), 53% (147.60 ± 11.66), and 49% (1.60 ± 0.11), respectively, as compared with the EST group.

**Serum Liver and Renal Functions.** Renal Functions. Table 2 shows a considerable rise in the serum creatinine levels and urea in the EST group by 93 and 33%, respectively, in comparison to the normal group. The protected (Gp 4) and treated (Gp 6) groups with ASLELN displayed significantly decreased levels of creatinine and urea by 57 and 51%, respectively, and by 62 and 21%, respectively, as compared to the EST group.

**Liver Functions.** Table 3 shows a considerable rise in the serum levels of ALP, ALT, AST, and total bilirubin in the EST group by 33, 21, 86, and 27%, respectively, with a significant decrease in albumin by 26% in comparison to the normal group. The protected (Gp 4) and treated (Gp 6) groups with ASLELN displayed significantly decreased levels of ALT, AST, ALP, and total bilirubin by 20, 16, 36, and 17%, respectively, and by 20, 14, 33, and 18%, respectively, as compared to the EST group and, notably, an increase in albumin level by 28% in the protected group with ASLELN (Gp 4) only.

**Oxidative and Inflammatory Markers.** Ehrlich Tumor Homogenate. As illustrated in Figure 6, the tumor reduced glutathione (GSH) content (a) and superoxide dismutase (SOD) activity (c) of the normal group was (13.39 ± 0.12 and 110.60 ± 2.78) and significantly decreased in the EST group by 27% (9.80 ± 0.18) and 26% (81.44 ± 2.60) respectively as compared with normal rats. Nevertheless, the protected (Gp 4) and treated (Gp 6) groups with ASLELN displayed significantly boost GSH, and SOD by 25% (12.29 ± 0.64), 30% (12.76 ± 0.33), and 33% (108.20 ± 3.05), 26% (102.40 ± 3.35) respectively as compared to EST group.

The tumor MDA (b), NO (e) contents, and MPO activity (d) of the normal group were (23.90 ± 1.96, 58.10 ± 1.67, 0.45 ± 0.02) respectively. They significantly increased in the EST group by 184% (67.98 ± 3.47), 59% (92.59 ± 4.63), and 542% (2.89 ± 0.13) respectively as compared with normal rats.

As compared with the EST group, the tumor MDA, NO, and MPO significantly decreased in the protected group with ASLELN (Gp 4) by 50% (2.60 ± 0.12), 39% (56.25 ± 1.20), 77% (0.67 ± 0.04) respectively but in the treated group with ASLELN (Gp 6), only MDA significantly decrease by 30% (47.43 ± 2.91) with no significant change in both NO and MPO.

**Hepatic Homogenate.** Table 4 showed a significant decrease in the hepatic GSH content and SOD activity in the EST group by 43 and 36% respectively with significant raise in hepatic MDA, NO content in companies with MPO activity by 150, 88, and 98% respectively in comparison to the normal group. While the protected (Gp 4) and treated (Gp 6) groups with ASLELN displayed significantly decreased MDA, NO, and MPO by 50, 30, 48% respectively, and by 47, 22, 43% respectively as compared to the untreated EST group plus notably increase in GSH and SOD by 57 and 42% respectively in the protected group with ASLELN (Gp 4) only.

**Renal Homogenate.** Table 5 showed a significant decrease in the renal GSH content and SOD activity in the EST group by 34 and 34% respectively with significant raise in renal MDA, NO content in companies with MPO activity by 200, 227, and 137% respectively in comparison to the normal group. While the protected (Gp 4) and treated (Gp 6) groups with ASLELN displayed significantly decreased MDA, NO, and MPO by 53, 20, 49% respectively, and by 46, 22, and 39% respectively as compared to the untreated EST group plus notably increase in GSH and SOD by 42 and 30% respectively in the protected group with ASLELN (Gp 4) only.

**Histopathology.** The histopathology of the liver, kidney, and tumor tissues in the mice is studied to reveal the treatment effect of free and ASLELN. As shown in Figure 7 Gp 1 has liver tissue with the normal architecture of hepatocytes around the central vein, kidney tissue with normal renal tissue, and skeletal

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**Table 2. Effects of ASLE and ASLELN on Renal Markers in Mice Serum Bearing the Ehrlich Solid Tumor (EST)**

| groups       | creatinine (mg/dL) | urea (mg/dL) |
|--------------|--------------------|--------------|
| normal       | 0.56 ± 0.02        | 61.87 ± 1.74 |
| EST          | 1.64 ± 0.06        | 82.15 ± 5.13 |
| ASLE pre-EST | 0.70 ± 0.06        | 49.46 ± 0.67 |
| ASLELN pre-EST | 0.69 ± 0.05      | 40.50 ± 5.50 |
| EST with ASLE | 0.76 ± 0.03        | 56.67 ± 2.71 |
| EST with ASLELN | 0.63 ± 0.03    | 64.49 ± 3.41 |

*Each value indicates the mean ± SEM. Significantly different from the normal group at p < 0.05. Significantly different from the control EST group at p < 0.05. Statistical analysis was performed by one-way ANOVA followed by Tukey multiple comparison test.*

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**Table 3. Effects of Tested ASLE and ASLELN on Liver Functions in Mice Serum Bearing the Ehrlich Solid Tumor (EST)**

| Groups       | ALT (U/L) | AST (U/L) | ALP (U/L) | ALB (g/dL) | TP (g/dL) | total bilirubin (mg/dL) |
|--------------|-----------|-----------|-----------|------------|-----------|------------------------|
| normal       | 17.75 ± 0.66 | 15.30 ± 0.10 | 48.23 ± 3.95 | 3.02 ± 0.05 | 6.80 ± 0.41 | 1.03 ± 0.01 |
| EST          | 23.63 ± 0.57 | 18.50 ± 0.32 | 89.60 ± 3.98 | 2.24 ± 0.02 | 5.36 ± 0.18 | 1.31 ± 0.01 |
| ASLE pre-EST | 21.88 ± 0.33 | 16.53 ± 0.16 | 73.50 ± 4.60 | 2.59 ± 0.17 | 6.09 ± 0.19 | 1.19 ± 0.01 |
| ASLELN pre-EST | 18.88 ± 0.25 | 15.53 ± 0.20 | 57.40 ± 2.30 | 2.87 ± 0.18 | 6.78 ± 0.43 | 1.09 ± 0.02 |
| EST with ASLE | 19.25 ± 0.45 | 16.27 ± 0.53 | 65.27 ± 5.28 | 2.79 ± 0.15 | 6.72 ± 0.14 | 1.07 ± 0.03 |
| EST with ASLELN | 19.00 ± 0.33 | 15.93 ± 0.14 | 59.70 ± 4.60 | 2.78 ± 0.11 | 6.58 ± 0.35 | 1.07 ± 0.03 |

*Each value indicates the mean ± SEM. Significantly different from the normal group at p < 0.05. Significantly different from the control EST group at p < 0.05. Statistical analysis was performed by one-way ANOVA followed by Tukey multiple comparison test.*
muscle tissue with normal muscle fibers striation. Figure 7 Gp 2 showed liver tissue with congested central vein with infiltration of activated Kupffer’s cells, a glomerular tuft of the kidney with slightly hyperplastic glomerular cells, and skeletal muscles tissue with the invasion of the neoplastic cells to the surrounding skeletal muscles with marked necrosis (H&E, 50 μm). Figure 7 Gp 3 has hepatic tissue of central vein with infiltration of activated Kupffer’s cells, the obliteration of Bowman’s space in the kidney tissue with the hyperplastic glomerular cells with dilatation and congestion of renal blood vessel and muscle bundles with the invading neoplastic cells diffusing all around its adjacent tissue with marked necrosis. Gp 4 has hepatic tissue between hepatocytes, kidney tissue with the obliteration of Bowman's space with the hyperplastic glomerular cells with dilatation and congestion of renal blood vessel, and the neoplastic cells diffusing all around its adjacent tissue with necrosis of skeletal muscles. Gp 5 showed liver with congested

Figure 6. Levels of oxidant and antioxidant markers (a) GSH content, (b) MDA, (c) SOD activity, (d) MPO activity, and (e) NO content in tumor homogenate of different studied groups. Each value indicates the mean ± SEM. *Different significantly from the normal group at p < 0.05. #Different Significantly from the control EST group at p < 0.05. One way of exhibiting statistical analysis was performed by ANOVA followed by Tukey Multiple Comparison Test.

Table 4. Effects of Tested ASLE and ASLELN on Hepatic Oxidative and Inflammatory Markers in Ehrlich Solid Tumor (EST)-Bearing Mice

| groups        | GSH (mmol/g tissue) | SOD (U/g protein) | MDA (mmol/g tissue) | NO (μmol/g tissue) | MPO (U/g tissue) |
|---------------|---------------------|-------------------|---------------------|--------------------|------------------|
| normal        | 30.00 ± 1.92        | 138.80 ± 3.61     | 31.11 ± 2.03        | 24.08 ± 1.40       | 28.32 ± 1.76     |
| EST           | 17.22b ± 0.55       | 89.19b ± 3.24     | 77.55b ± 2.31       | 45.37b ± 1.80      | 56.19b ± 1.59    |
| ASLE pre-EST  | 23.70bc ± 0.74      | 108.90bc ± 2.30   | 44.69bc ± 2.70      | 41.20bc ± 0.61     | 48.96bc ± 2.12   |
| ASLELN pre-EST| 27.04bc ± 0.22      | 126.80bc ± 2.09   | 38.84bc ± 2.46      | 31.71bc ± 1.61     | 29.10bc ± 1.41   |
| EST with ASLE | 19.61b ± 0.45       | 104.50b ± 2.65    | 51.70bce ± 3.47     | 36.11bce ± 0.69    | 34.33b ± 1.87    |
| EST with ASLELN| 21.66bc ± 0.96      | 112.60b ± 4.28    | 40.76b ± 3.38       | 35.41bc ± 1.44     | 32.21b ± 0.93    |

Each value indicates the mean ± SEM. *Different significantly from the normal group at p < 0.05. #Different Significantly from the control EST group at p < 0.05. One way of exhibiting statistical analysis was performed by ANOVA followed by Tukey Multiple Comparison Test.
Table 5. Effects of Tested ASLE and ASLELN on Renal Oxidative and Inflammatory Markers in Ehrlich Solid Tumor (EST)-Bearing Mice

| groups | GSH (μmol/g tissue) | SOD (U/g protein) | MDA (μmol/g tissue) | NO (μmol/g tissue) | MPO (U/g tissue) |
|--------|---------------------|-------------------|--------------------|-------------------|------------------|
| normal | 21.11 ± 0.55        | 128.40 ± 4.52     | 24.10 ± 1.34       | 18.15 ± 0.77      | 48.84 ± 3.21     |
| EST    | 13.89 ± 0.55        | 84.50 ± 4.31      | 72.51 ± 1.88       | 59.32 ± 2.19      | 115.60 ± 5.51    |
| ASLE-1  | 13.89 ± 0.48    | 99.54 ± 1.99      | 41.79 ± 2.28       | 51.85 ± 2.13      | 110.80 ± 6.92    |
| ASLELN  | 19.75 ± 1.30      | 110.20 ± 7.56     | 34.17 ± 2.35       | 47.53 ± 1.63      | 58.40 ± 4.59     |
| EST with ASLE | 16.79 ± 0.99 | 96.85 ± 1.81      | 42.59 ± 3.44       | 50.00 ± 2.13      | 109.70 ± 5.40    |
| EST with ASLELN | 17.58 ± 0.88 | 103.00 ± 4.76     | 39.12 ± 1.47       | 46.30 ± 2.82      | 68.13 ± 3.85     |

*Each value indicates the mean ± SEM. a Different significantly from the normal group at p < 0.05. b Different significantly from the control EST group at p < 0.05. One way of exhibiting statistical analysis was performed by ANOVA followed by Tukey Multiple Comparison Test.

中央静脉と活性化したKupfferの細胞が浸潤し、腎臓、肝臓の辺縁部、筋肉の細胞に加えて、腫瘍細胞が自身の周囲に広がる。この進展が腎臓の血流を阻害し、筋肉の成長を減じることになる。

## DISCUSSION

The challenging goal of cancer research continuously is discovering safe and effective new natural and synthetic products with anti-cancer effects. Plant-derived natural products have incredible potential to control cancer cell growth. Previous studies confirmed that the ASLE contained flavonoids, alkaloids, saponins, tannins, glycosides, steroids, phenols, and various acetogenin compounds and demonstrated a variety of pharmacological activities involving antioxidant, anti-cancer, and anti-inflammatory properties. Nowadays, plants are performing a remarkable role in nanoparticle synthesis. To ameliorate cancer therapeutic protocols, the current study was implemented to determine the antitumor efficacy and hepato-renal protective effect of ASLELN against Ehrlich Ascites Carcinoma (EAC) in mice.

Figure 3 showed the release profile for niosomes loaded ASLE could be described as a two-step release process, involving an initial burst release (first hour), which could be due to diffusion of the compounds adsorbed on the niosomes surface, followed by subsequent slower release (after 10 h) and this could be attributed to the diffusion of A. squamosa leaves extract dispersed into the niosomes inner layers.

Ehrlich tumor is a type of cancer that grows progressively quickly with a very destructive effect that can evolve in almost all strains of mice. Nevertheless, it is commonly used as an experimental animal model for a tumor because of its moderate cost, easy induction, effective reproducibility, and availability to assess the safety and efficacy of anticancer therapies. Reactive oxygen species (ROS) and other free radicals are the most potent mediators of cellular toxicity, particularly in cancer. Several studies have shown that tumor growth results in the disturbance of the antioxidant system and the production of lipid peroxidation in pivotal organs of the tumor-host. In external stress and carcinogenesis, the increase in macrophage-produced ROS, that increased lipid peroxidation which prompted immune response and induced the transcriptional factor NF-κB. Also, inflammation induces degradation of the inhibitory protein (IkB) that connects to the inactivated form of NF-κB in the cytoplasm, resulting in

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Figure 7. Histopathology photomicrographs for mic (liver, kidney, and skeletal muscles) tissues. Group 1 showed: liver tissue with the normal architecture of hepatocytes around the central vein (H&E, 10 μm), kidney tissue with normal renal tissue (H&E, 50 μm), skeletal muscles tissue with normal muscle fibers striation (H&E, 50 μm). Group 2 showed: liver tissue congested central vein with infiltration of activated Kupffer’s cells (H&E, 50 μm), a glomerular tuft of the kidney with slightly hyperplastic glomerular cells (H&E, 25 μm), skeletal muscles tissue with the invasion of the neoplastic cells to the surrounding skeletal muscles with marked necrosis (H&E, 50 μm). Group 3 showed: hepatic tissue of the central vein with infiltration of activated Kupffer’s cells (H&E, 10 μm), the obliteration of Bowman’s space in the kidney tissue with the hyperplastic glomerular cells (white arrows) with dilatation and congestion of renal blood vessel (black arrow) (H&E, 50 μm), muscle bundles with the invading neoplastic cells diffusing all around its adjacent tissue with marked necrosis (H&E, 50 μm). Group 4 showed: hepatic tissue with infiltration of activated Kupffer’s cells between hepatocytes (H&E, 50 μm), kidney have the obliteration of Bowman’s space with the hyperplastic glomerular cells with dilatation and congestion of renal blood vessel (H&E, 50 μm), the neoplastic cells diffusing all around its adjacent tissue with necrosis of skeletal muscles (H&E, 50 μm). Group 5 showed: that the liver has congested central veins with an activated Kupffer’s cells infiltrating in between (H&E, 50 μm), kidney with the obliteration of Bowman’s space with the hyperplastic glomerular cells with dilatation and congestion of renal blood vessel (H&E, 50 μm), muscle bundles with the invading neoplastic cells diffusing all around its adjacent tissue with marked necrosis (H&E, 10 μm). Group 6 showed: Image of the liver with the normal architecture of the hepatic lobules (H&E, 50 μm), Normal renal tissue architecture (H&E, 50 μm), and muscle bundles showing the invading neoplastic cells diffusing all around its adjacent tissue with marked necrosis (H&E, 10 μm).

On the other hand, our results showed the anti-inflammatory activity of ASLE and ASLELN by decreasing NO and MPO levels, especially in Gp (4) get better-ameliorated effects. This may be due to the variety of bioactive properties of the polyphenolic compounds and flavonoids as quercetin content of these extracts. One of the most important antioxidant mechanisms of polyphenols is the regulation of enzyme activity, as inhibition of oxidases such as cyclooxygenase results in a reduction in the production of ROS in vivo. In addition, flavonoid derivatives such as quercetin can exert multiple pharmacological activities including anti-inflammatory activity by minimizing the inflammatory factors production as NF-κB, TNF-α, and COX-2 in Human Colon Cancer Caco-2 Cells. Nitric oxide and the pro-oxidant enzyme myeloperoxidase (MPO) were playing a fundamental role in controlling cellular stress and constituting a key component of the innate immune defense system. Excessive production of NO and MPO-derived oxidants have been associated with tissue damage as well as the onset and the progression of chronic inflammation-related disorders including cancer involved in EAC-induced toxicity, this agrees with our findings that demonstrated a significant increase in NO level and MPO activity in the selected organs. Our results are in line with ELAblack et al. (2020) that explained the increase of NO level may be due to the elevation of lipid peroxidation levels and its product MDA that can enhance host cells, fundamentally macrophages/monocytes, to produce NO by stimulation of iNOS activity causing tissue damage. Also, overproduction of TNF-α may be the reason for the elevation of NO concentration, where TNF-α acts as a mediator of NO synthesis. Besides that, the elevation of MPO activity may be attributed to the high levels of ROS that are produced in several human tumors.

The best criteria for judging the efficacy of any anticancer agents are the reduction of solid tumor volume and the vanishing of leukemic cells from the blood. Previous studies reported that the perturbation in the inflammatory regulators
such as TNF-α and NO resulted in tumor proliferation and growth, which was elucidated by the increase in the solid tumor volume.\textsuperscript{5,3} The current study showed that the inoculation of EAC cells caused a significant increase in solid tumor volume due to cell multiplicity and the development of EAC in the EST-bearing group.\textsuperscript{4,34} Pre or with the administration of ASLE or ASLELN caused a distinct reduction in the solid tumor volume, with a more pronounced decrease in the treated EST-bearing mice with ASLELN. These results elucidated the antitumor efficacy of the extracts, particularly nanoforms that might be credited to the presence of flavonoid and phenolic compounds which may activate macrophages and inhibit vascular permeability.\textsuperscript{55} It may be pointed out that, the presence of annoneconjugated acetogenins compounds as a component of \textit{A. squamosa} leaves extract\textsuperscript{56} may contribute to the antitumor activity of these extracts.\textsuperscript{57} Our results are consistent with Wang et al. (2014)\textsuperscript{58} who reported that ethyl acetate extract of \textit{A. squamosa} leaves had anticancer activity on colon cancer and human epidermoid carcinoma cell line.

The tumor growth was accompanied by hematological changes which encompass a decrease in RBCs count, hemoglobin content, and an increase in WBC count, neutrophils, and monocytes. The correlation between anemia and cancer progression was reported in epidemiological studies.\textsuperscript{59} The main reason for this anemia is iron deficiency which causes the reduction of hemoglobin content of RBCs.\textsuperscript{60} The elevation of WBC count in EST-bearing mice may be attributed to the activation of bone marrow.\textsuperscript{61} ASLE or ASLELN supplementation especially group (4) maintained the normal values of WBC and RBC count and replenishes hemoglobin content, which proposes its hematopoietic protecting action. These results are following the previous study.\textsuperscript{31} Manthou et al. (2017)\textsuperscript{62} explained that the presence of flavonoids and polyphenolic compounds increased antioxidant capacity in erythrocytes which prevents oxidation of hemoglobin and RBCs destruction by reactive species. The anti-inflammatory and antioxidant activity of the extracts could explain their hematopoietic activity.\textsuperscript{18}

The vital functions of organs can be inversely affected by tumor growth, regardless of the tumor site.\textsuperscript{63} Cancer causes liver and kidney dysfunction and interferes in hepatic cell metabolism resulting in alterations in serum enzyme activity.\textsuperscript{64} In our investigation, elevated serum levels of ALP, ALT, AST, total bilirubin plus serum creatinine and urea levels, with a concomitant decrease in albumin elucidated a development of hepatotoxicity and renal tissue injury in EST-bearing mice. Hepatocytes are destroyed then the excess AST released into the bloodstream following the growth of the tumor may cause an elevation in liver enzymes.\textsuperscript{65} Besides that, the decline in the level of albumin produced by the liver’s hepatocytes in EST–bearing mice, valuable markers for liver function,\textsuperscript{66} refers to the harmful effect of EAC that causes the decrease in the biosynthetic capacity of the liver to synthesize plasma protein. Also, EAC proliferation induced renal tissue injury and raised kidney function.\textsuperscript{67} Our results are matched with a recent study.\textsuperscript{68,69} The changes in liver and kidney functions could be due to the generation of ROS that induces tissue damage by destroying cellular homeostasis.\textsuperscript{60} Our data indicated that treatment of EST–bearing mice with ASLE or ASLELN ameliorated the changes in the levels of the hepatotoxicity markers and kidney functions indicating the potent hepatorenal protective effect of these extracts through its scavenging and antioxidant characteristics due to its flavonoid and phenolic compounds contents.\textsuperscript{70} These findings were consistent with previous results.\textsuperscript{32,71,72}

\section*{CONCLUSIONS}
\textit{A. squamosa} leaf extracts could aid in the creation of new pharmaceuticals as a source of alternative chemotherapy for sarcoma cancer treatments. As niosomes nanocarriers are cheap, non-toxic, biocompatible, and release drugs with proper control, so this study loads \textit{A. squamosa} leaves extract onto niosomes to raise its therapeutic efficacy in cancer-infected mice. \textit{A. squamosa} leaves extract-loaded niosomes and free extract reduced tumor size and improved liver and kidney functions, inflammatory markers, oxidative stress, and hematological parameters relative to positive control. There was no discernible difference between the effectiveness of these two tested formulations in terms of the levels of biochemical markers, however, the extract-loaded niosomes were more effective than the free extract at shrinking tumors. Therefore, in addition to cancer patients, \textit{Annona squamosa} leaf extracts can be recommended to individuals who have high-risk factors for developing cancer. The molecular mechanisms that \textit{A. squamosa} leaf extracts target cancer cells require further study.

\section*{EXPERIMENTAL SECTION}
\textbf{Chemicals.} Fisher Scientific U.K. provided Methyl alcohol. Phosphate buffer tablets pH 7.4 (purity >98\%) was purchased from Bio Shop Canada Inc. Absolute Ethanol, Tween 80 (purity ≥99\%), and cholesterol (M, 386.65, purity ≥99.7\%) were purchased from Sigma Aldrich.

\textbf{Plant Materials and Preparations.} \textit{Plant Material.} Fresh plant materials were collected (March 2021) from a local plant farm at Mansoriya, Giza, Egypt. Plant taxonomists identified and authenticated the herb as \textit{A. squamosa} L. at the Herbarium, Desert Research Center, Cairo, Egypt. Fresh leaves of the plant were washed with running tap water to clean, shade-dried at room temperature for 7 days, and were finely ground using an electric blender. The obtained powder was stored in a dry closed container and kept at −20 °C in the dark until used.\textsuperscript{10}

\textbf{Plant Extraction.} About 770 g of \textit{A. squamosa} leaves (ASL) powder were weighed accurately and soaked in 1500 mL of 70\% methanol and then kept in a dark place for 3 days, carbon dioxide was released frequently. The resulting aqueous fraction was filtered through filter paper. The filtrate was concentrated under vacuum by a rotary evaporator at about 40 °C. The concentrated \textit{A. squamosa} leaves extract (ASLE) was collected and stored to use later.

\textbf{Niosomes Preparation and Characterization.} \textit{Preparation.} Ethanol was used to dissolve tween, cholesterol, and the extract in a rounded flask, then the ethanol was evaporated at 45 °C to form dried thin layers on the flask inside the wall. Niosomes were formed by hydration of dried thin layers with phosphate-buffered saline (PBS) (pH 7.4), then subjected to sonication (WUC-A03H, Daihan, Korea) for 5 min to produce small niosomes vesicles. A cooling centrifuge (11,000 rpm for 30 min) (vision scientific 18,000 M, Korea) was used to precipitate niosomes.\textsuperscript{3}

\textbf{Characterization.} \textit{Loading Efficiency.} Different known concentrations of ASL extract were evaluated by UV–visible spectrophotometry (JENWAY Scanning, 6405, United Kingdom) at a wavelength of 270 nm. ASL extract standardization curve was created by plotting absorbance versus concentration
of ASL extract. The initial concentration was evaluated for ASL extract before loading in niosomes. The efficiency of the A. squamosa leaves extract-loaded noisome (ASLELN) was achieved by the centrifugation technique.\(^7\) The loaded niosomes suspended in PBS were centrifuged (11,000 rpm, 30 min, VS-18000 M, Korea) to isolate the free extract from the niosomes. The isolated free ASL extract concentration was measured spectrophotometrically using the standard curve to get the final concentration. Then the loading efficiency of niosomes for ASL extract was evaluated from the next equation

\[
\text{loading efficiency} = \frac{\text{initial concentration} - \text{final concentration}}{\text{initial concentration}} \times 100
\]

**UV Spectrum.** The absorption of the ASLE and ASLELN was determined by a UV spectrophotometer over the range of 200–1000 nm to confirm the loading process.

**Transmission Electron Microscope.** The transmission electron microscope (JEOL JEM-1230, Japan) was used to illustrate the niosomes structure. The accelerating voltage was 102 kV to perform this evaluation. Niosomes suspension-loaded by the extract was negatively stained by 1% of phosphotungstic acid. Samples were incubated in grids of perforated carbon-coated for 10 min after the dray had been running for 5 min.\(^3\)

**In Vitro Extract Release.** The dialysis technique was used to determine the ASL extract release from niosomes in PBS (pH 7.4). Briefly, 3 mL of ASLELN were placed in a dialysis bag (cellulose acetate Spectra/Por, MWCO-12,000, Spectrum, Canada) and were dived in 100 mL phosphate buffer saline containing 1 mL tween 20 to enhance the extract solubility on the magnetic stirrer (50 rpm, TK22-Kartell, Italy). Every 1 h, the absorbance of 2 mL of immersing solution was measured by UV—visible spectrophotometry at 270 nm.

**Animals.** In total, 72 male albino mice (weighing 25 ± 2 g) were obtained from the National Research Centre, Cairo, Egypt. Mice were kept in mesh steel wire cages with adjustable temperature controls set at (22 ± 3) °C, light (dark/light cycles every 12 h), and (80 ± 4) % relative humidity, as well as free access to food (diet and water). All procedures were carried out according to the research ethics committee for experimental studies at Cairo University Institutional Animal Care and Use Committee (CU-IACUC), based on verification of application number CU/1/F/27/21.

**Tumor Inoculation.** Ehrlich ascites carcinoma (EAC) cells were obtained from EAC-bearing mice provided by Cairo University’s Egyptian National Cancer Institute (NCI). 0.2 mL of ascetic fluid from EAC mice were aspirated and diluted in physiological buffer saline. EAC cells were counted before being subcutaneously injected into the left thigh of the mouse’s lower limb to induce a solid tumor in the experimental mice.

**Experimental Design.** One week before the experiments, all mice were kept in a normal laboratory environment for acclimatization, and then they were divided randomly into six equal groups (12 mice per group) as follows:

- **Group 1:** Normal mice without any treatment and served as a negative control group.
- **Group 2 (EST):** Mice were injected subcutaneously with about 2.5 to 3 × 10^6 EAC cells/mouse diluted in physiological saline.\(^4\) Mice were incubated for 24 h, then received saline orally for 24 successive days, and served as a positive control group (EST-bearing group).

- **Group 3 (Extract + EST):** Before the injection of EAC cells, mice received ASLE orally once daily (250 mg/kg) for 1 week. The oral administration of ASLE was subsequently continued for an additional 24 days (ASLE pre-EST).

- **Group 4 (Nanoform + EST):** Before the injection of EAC cells, mice were given ASLELN orally once daily for 1 week (250 mg/kg), following which the oral administration of ASLELN was continued for an additional 24 days (ASLELN pre-EST).

- **Group 5 (EST + Extract):** Mice were injected with EAC cells, incubated for 24 h, and then given ASLE orally once daily (250 mg/kg) for 24 successive days. (EST with ASLE).

- **Group 6 (EST + Nanoform):** Mice were injected with EAC cells, incubated for 24 h, and then given an oral dose of ASLELN once daily (250 mg/kg) for 24 successive days (EST with ASLELN).

At the end of the experiment, 24 h after the last manipulation and overnight fasting, samples of the blood were obtained and collected from mice’s retro-orbital venous plexus into two separate tubes; the first tube contained EDTA to quantify the hematological indices, the second was clean centrifuge tube and the blood left to coagulate for 20 min at 37°C then centrifuged (for 20 min at 3000 rpm) to separate serum. The serum was stored (at −80°C) for further analysis. The biochemical markers used in our analysis were albumin, urea, creatinine, total bilirubin, total protein, ALT, ALP, and AST in addition to other inflammatory markers such as (TNF-α, COX-2, and NF-kB). Thereafter, animals were euthanized by decapitation. Solid tumors, the right thigh of the lower limb, liver, and kidney from the control and the treated groups were excised and washed with cold sterile isotonic saline. To create the 10% homogenate that will be utilized for the evaluation of oxidative stress biomarkers (GSH, SOD, MDA) and inflammatory indicators (MPO, NO), solid tumors, liver, and kidneys were weighed and homogenized in ice-cold PBS. Some fragments from the normal muscles, tumor, liver, and kidneys were kept in 10% neutral buffered formalin for histopathological investigation while the other specimens were stored at −80°C for biochemical analysis.

**Determination of Solid Tumor Volume.** Tumor growth was regularly measured twice a week for 2 successive weeks from the 11th day after the tumor induction. The length and width of tumors were measured by vernier calipers. The volume of the tumor mass (V) was calculated by using the following equation

\[
\text{tumor volume (V)} = \frac{4}{3} \pi (a)^2 b
\]

\(^7\) Where (a) is the length of the major tumor axis, and the height and width are equal to the minor axis (b). The constant value (0.4) represents the linear regression coefficient.

**Biochemical Analysis. Hematological Parameters.** A fresh blood sample (0.25 mL) was added to 20 mg EDTA to investigate the hematological parameters such as Hb (Hemoglobin) content, RBCs, WBCs, and the percentage values of differential white cell count (Neutrophils, Lymphocytes, and monocytes).

**Determination of Serum Kidney and Liver Functions.** The separated serum was used to evaluate ALP (alkaline phosphatases), ALT (alanine aminotransferase), AST (aspartate aminotransferase) activities, total bilirubin, TP (total protein), Alb (albumin), urea, and creatinine levels by using commercially available kits obtained from Biodiagnostic company (Giza, Egypt).
Estimation of Inflammatory Biomarkers Markers. Using an in vitro enzyme-linked immunosorbent assay (ELISA) kit, the levels of Tumor necrosis factor-α (TNF-α; Abbexa, U.K.), nuclear factor-kappa (NF-kB; Sunlong Biotech Co., Ltd., Zhejiang, China), and cyclooxygenase-2 (COX-2; Bio Vision) were assayed in serum.

Estimation of the Inflammatory and Oxidative Stress Parameters. Malondialdehyde (MDA) was evaluated based on Uchiyama and Mihara’s (1978) method. Reduced glutathione (GSH) was measured. The activity of superoxide dismutase (SOD) was determined according to the method of Nandi and Chatterjee (1988). Determination of myeloperoxidase (MPO) activity was assessed according to the method of Bradley et al. (1982). Nitric oxide (NO) level was measured according to the method of Miranda et al. (2001).

Total protein was determined according to the method of Lowery et al. (1951).

Histopathological Examination. Following necropsy, the liver, kidneys, and Ehrlich solid tumors (EST) were immediately removed and preserved in 10% buffered neutral formalin for histological analysis. Paraffin-embedded sections of the solid EAC tumor, right thigh muscles of normal control, liver, and kidneys were sliced into a thickness of (4–5 μm) and stained with hematoxylin and eosin (H&E) solution and examined under a light microscope.

Statistical Analysis. All values in vivo results were presented as mean ± standard error of the mean (SEM). To evaluate the significance of the difference between treatments, one-way ANOVA was used, followed by the Tukey–Kramer multiple comparisons test. Values of P < 0.05 were considered significant. GraphPad PRISM program was used for statistical analysis (version 5, San Diego, CA).

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Notes
The authors declare no competing financial interest.

ABBREVIATIONS
ASL, A. squamosa leaves; ASLE, A. squamosa leaves extract; ASLELN, A. squamosa leaves extract-loaded niosomes; EAC, Ehrlich ascites carcinoma; EST, Ehrlich solid tumor

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