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New Strategy for Browning Prevention in Apple Pomace Processing and Toxicity Tested in a Rodent Model

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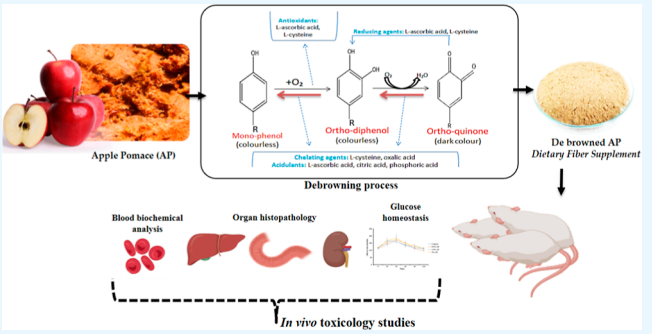
ABSTRACT: Apple pomace (AP) is a byproduct of apple juice industries, which constitutes around 30% of the original fruit and rich in essential compounds like carbohydrates, phenolics, minerals, and dietary fibers. This study is focused on optimizing the combinational utilization of antibrowning agents like L-cysteine and L-ascorbic acid for the sustainable debrowning of AP with response surface methodology and assessment of its acute toxicity in female rats. In addition, the phytochemical content and antioxidant activity of treated AP were investigated and compared with the untreated control AP. The study revealed that the treated AP has higher dietary fiber ($p < 0.01$), protein ($p < 0.0001$), and phenolic content ($p < 0.001$) in comparison to the control AP. Moreover, the treated AP also observed with higher antioxidant activity 37% inhibition and water retention capacity (8.5 g H_2O/g solid) along with the debrowning effect. Furthermore, a 4-week in vivo study is conducted to assess the toxicity of treated AP. Results indicated no discernible variations in biochemistry, morphometric, or histology between the supplementation (0.5, 1.5, and 3% AP) and control groups. Thus, adding AP rich in dietary fiber to a range of meals is deemed a safe and valuable food supplement.



1. INTRODUCTION

Apples are a fundamental ingredient in the preparation of numerous beverages, including juice, squash, and vinegar. Apple pomace (AP) is the byproduct of apple processing in apple juice industries (about 25−30% of the dry mass left after apple processing) and is one of the five fruit species accounting for the huge increase in world fruit production since 2000.[1] AP, comprising substantial levels of phytochemicals, carbohydrates, proteins, and essential vitamins (A, E, and C), along with minerals boasting significant antioxidant properties, stands as a valuable resource for sustainable reuse.[2] AP includes pulp, peel, seeds, calyx, and stem. Globally, an estimated 1.4 million metric tonnes of AP are generated annually.[3] Solvent extract of AP has a broad range of total phenolic content (TPC) concentration (262−856 mg of TPC/100 g). Fresh AP has a large proportion of water (70−85%) and sugar, making it wet and vulnerable to microbial degradation.[3] With up to 50% fermentable sugar concentration, dried AP has a high carbohydrate content. The primary sugars in AP are fructose (18−31%), sucrose (3.4−24%), and a small amount of glucose (2.5−12.4%).[4,5] The content of polyphenols in apples is dependent on numerous factors, such as the cultivar (due to different varieties such as Red Delicious, Golden Delicious, Ambri, Granny Smith, Rich Red, etc.), part of fruit, applied agronomic measures, climate conditions, maturity stage, harvesting, and method of processing.[6,36] The majority of the AP is composed of dietary fiber. It can be classified as soluble dietary fiber (13.5−14.6%) and insoluble dietary fiber (33.8−60.0%) based on how quickly they dissolve in water. AP contains concentrate of dietary fiber (mostly insoluble) because all the soluble components (sugar, acids, water, and soluble dietary fiber) are in the apple juice.[4] Cellulose, hemicellulose, and lignin make up insoluble dietary fibers. Apple pectin accounts for 10 to 15% of soluble dietary fiber on a dry weight.[5] Furthermore, diet supplemented with fiber is correlated with good digestive health, better glycemic control, and weight loss, as well as with reduction in gastrointestinal problems, coronary heart diseases, and prevention of certain type of cancer. In addition, AP inhibits lipid peroxidation and has radical scavenging capabilities.[7−9] Apple is a rich source of vitamins E and C compared to other fruits. According to reports, AP is a potential source of antioxidant molecules since it includes 22.4 mg/100 g of vitamin C. Polyphenols are significant secondary plant compounds that are mostly found in the topmost layer

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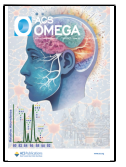

(skin), and AP has a high phenol concentration.[10] Apple browning from oxidation and fermentation is the largest problem encountered by apple processing industries. Browning immediately alters the color, flavor, and nutritional value of AP, making it unusable. Because it is heavy and expensive to transport, direct disposal of AP is another issue (rapid biodegradation by natural vegetation). The breakdown of AP increases the cost of manufacturing, increases greenhouse gas emissions, and endangers human health.[11] The fundamental cause of the browning of AP can be traced to polyphenol oxidase, which reacts with polyphenols to produce colored quinones and brown pigments. This enzymatic browning may be avoided by using chemical (acidulants, antioxidants or reducers, and chelating agents) and physical (blanching, drying, and freezing) methods. Therefore, preserving AP may maintain the vital nutrients or fiber material within its matrix by preventing browning and drying.[12] Sulfur-containing amino acids like L-cysteine, and glutathione, as well as L-ascorbic acid are known for having the strongest antibrowning properties. Instead of blocking the oxidation of phenolics by polyphenol oxidase, these amino acids prevented the subsequent polymerization of phenolics by producing colorless products containing *o*-quinones.[13]

Therefore, based on prior studies and owing to the many advantages of AP, including its high supply of dietary fibers, minerals, and antioxidants, it is possible that it will be used as a food supplement and functional food. To the best of our knowledge, a very limited number of studies were published on the use of a combination of antibrowning compounds and no one has used this combination of L-ascorbic acid and L-cysteine to avoid the browning effect on AP. Furthermore, limited cytotoxic studies have been reported and the combination of L-ascorbic acid and L-cysteine toxicity is not yet reported in previous literature. This new combinational approach is reported for the first time in this study.

This study optimizes the sustainable approach to antibrowning agents for AP and assesses their acute toxicity in female Sprague-Dawley rats. Response surface methodology (RSM) used to optimize the sustainable combination of antibrowning agents, L-ascorbic acid and L-cysteine treatment of AP. Furthermore, its phytochemical content, antioxidant activity, color, water retention, and surface morphology were studied with optimized antibrowning treatments. In addition, a 4-week in vivo study was carried out to compare the biochemistry, morphometry, and histology of treated AP to those of control as per the FDA Redbook 2000:IV.C.3. a guideline.[14]

2. MATERIALS AND METHODS

2.1. Apple Pomace. AP containing pulp residue (a byproduct of the fruit-juice industry)[3,15] was obtained after the juice was extracted and filtered. AP belongs to the Royal Delicious family and was procured from Punjab Agri Export Corporation Limited, Village Jahan Khelan, District Hoshiarpur (Punjab), India, after processing of cloudy apple juice, dated on February 2024. The material was kept at −20 °C until further examination.

2.2. Chemicals and Solvents. All chemical reagents were purchased from Sigma-Aldrich, St. Louis, Missouri, United States (L-cysteine, copper-sulfate pentahydrate, ethanol, formaldehyde, and methanol), Chemical Drug House, New Delhi, India (L-ascorbic acid, citric acid, oxalic acid, phosphoric acid, petroleum ether, acetic acid, hydrogen peroxide, sodium hexametaphosphate, sodium hypochlorite, sodium hydroxide, sodium carbonate, sodium potassium tartrate, Folin-Ciocalteu's phenol reagent, hydrochloric acid, sodium phosphate monobasic, sodium phosphate dibasic, and chloroform) and Sisco Research Laboratories, Mumbai, India [DPPH (2,2-diphenyl-1-picrylhydrazyl)]. Deionized water was used in the preparation of solutions and buffers.

2.3. Antibrowning Treatments. *2.3.1. Screening of Antibrowning Agents.* AP (pulp) was immersed in a solution (1:10) containing different antibrowning chemicals in varying proportion with double-distilled water in order to reduce the beginning of browning. The most commonly used antioxidant is an ascorbic acid, which shows antibrowning properties by reducing the oxidized substrate and product *ortho*-quinones back to diphenols.[16,17] It is irreversibly oxidized to dehydroascorbic acid during the reduction process. However, the oxidation ability of the L-ascorbic acid of reducing quinone is related to its concentration and since the applied concentration is consumed during the process, its antibrowning effect could be time limited.[12] Thus, it has been studied in conjunction with other preservatives. The different combinations of inhibitors included were as follows: L-ascorbic acid (2%), phosphoric acid (1%);[12] L-ascorbic acid (2%), citric acid (1%);[18] L-ascorbic acid (2%), oxalic acid (1%), acetic acid (1%);[12] L-ascorbic acid (2%), citric acid (1%), acetic acid (1%); L-ascorbic acid (2%), citric acid (1 and 2%), hydrogen peroxide (1%); L-ascorbic acid (1%),[19] L-cysteine (1%), sodium hypochlorite (4 ppm);[17] L-ascorbic acid (2%), citric acid (1%), sodium hexametaphosphate (1%);[20] L-ascorbic acid (2%), L-cysteine (1%), citric acid (1%); L-ascorbic acid (1%), L-cysteine (1%);[16] and L-ascorbic acid (2%), L-cysteine (1%). The mixture of pomace and inhibitors were homogenized at 300 rpm for 3−5 min and below 4 pH was maintained.[12,20] Throughout the procedure, all of the samples were visually examined. L-ascorbic acid, L-cysteine, and their combination were then further examined utilizing the RSM.

2.3.2. Optimization of Pretreatment. The experiment data were analyzed using RSM and utilized to design trials for the debrowning of AP. The results and statistical analysis of the experimental data were analyzed using analysis of variance (ANOVA) and using Design Expert statistical software (version 12.0.11.0, Stat-Ease, Inc., Minneapolis, USA). A 2^n factorial central composite design was used, with six replicates at the center points producing 32 runs (Table S1). Every independent variable's low (−1) and high (+1) values were determined as follows: L-ascorbic acid, % (0, 2); L-cysteine, % (0, 2); mixing time, min;[5,25] temperature, °C;[20,40] and rotation per minute, rpm (200, 400) and were categorized as *A*, *B*, *C*, *D*, and *E*, respectively, against two responses, i.e., yield (%) and color (L^*-value). The L^*-value connotes browning and thus can be used to describe possible enzymatic reactions.[18] The yield (%) was calculated by dividing treated AP against controlled AP, after pretreatment and vacuum drying. Since, drying results in the removal of excess water content, thus increasing shelf life by reducing the spoilage, so calculating yield (%) can be helpful to determine further utilization of AP on a commercial scale. The F test was used to estimate the variance related to the ANOVA pure error variance. After eliminating the insignificant interactions ($p > 0.05$), based on the coded levels of the independent variables, an RSM was fitted. The coefficient of determination served as a measure of how well the model matched the data (R^2). According to DOE (Table S1), 10 g of AP was combined with 1:10 (w/v) solution

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of L-ascorbic acid and L-cysteine for each experiment and mixing duration, temperature, and rpm were adjusted.

2.4. Dehydration of AP. The debrowned AP was dried using a vacuum oven drier (OV-12, Jeiotech, UK). The oven was warmed to 70 °C before the samples were placed in the chamber. The pressure inside the oven was maintained by a preinstalled vacuum pump which helped in reducing the pressure to 0.08 MPa. The material for analysis was equally dispersed in a 2 mm layer and stored for around 2.30−3 h. The vacuum-dried material was then grinded using laboratory mill PX-MFC 90D polymix, Kinematica AG, Switzerland, and passed through mesh size 0.5 mm.

2.5. Physical Properties of Apple Pomace (AP). *2.5.1. Color Measurement.* The color of the dried debrowned AP was measured with a colorimeter (Lovibond LC 100/SV 100 hand-held Spectro Colorimeter, England). The instrument was calibrated with a white standard plate. The powder was placed in an optical cell and the parameters estimated were L^*, a^*, and b^*-values which range from darkness (0) to lightness (100), red to green, and blue to yellow, respectively. The results were stated in relation to the Hunter Lab color CIE L^*a^*b space.[21,22]

2.5.2. Estimation of Water Holding Capacity. Water holding capacity (WHC) was estimated by the method described previously[23] with slight modifications. One gm of the debrowned AP was mixed with 30 mL water and incubated at room temperature (25 °C) for 24 h. Final weight of residual was measured and WHC was calculated using the below given formula as described

$$\text{WHC} = \frac{W2 - W1}{W1}$$

Here, $W1$ = initial sample weight and $W3$ = final weight of the sample.

2.5.3. Scanning Electron Microscopy. Morphological characterization of control (AP) and treated (debrowned AP) was carried out using a JEOL JCM-6000 Benchtop scanning electron microscope (JEOL Ltd., Tokyo, Japan). The test sample particles having a diameter of less than 100 μm were fixed on an aluminum stub and the final scanning electron microscopy (SEM) images were viewed at 10 kV acceleration voltages and 50× magnification.

2.6. Nutritional Composition of AP Powder. *2.6.1. Estimation of Moisture Content.* The moisture content of samples was done using the MA35 Sartorius Moisture Analyzer (Sartorius Weighing Technology GmbH, Goettingen, Germany). The test samples were heated at 105 °C to make constant residual weight.[24] When there is no further change in weight of the sample, the equipment indicates the "END" point and thus final % moisture was recorded.

2.6.2. Estimation of Ash Content. The ash content of the sample was analyzed by the method described previously withy slight modifications[25] and was heated at 550 °C for 4 h in an oven. After ashing, samples were cooled down and weighed until two consecutive readings remain constant. The percentage ash content was evaluated as

$$\text{ash}\ (\%) = \frac{(W3 - W2) \times 100}{(W2 - W1)}$$

where $W1$ = empty silica crucible weight, $W2$ = sample + silica crucible weight, and $W3$ = ash + silica crucible weight.

2.6.3. Estimation of Fat Content. The fat content was calculated using the Soxhlet apparatus. The samples were extracted in Soxhlet apparatus at for 6 h at 40 °C using petroleum ether as a solvent.[25] The resultant ether extracts were collected in a flat bottom flask and evaporated to remove any traces of ether using a rotary evaporation system (Cole-Parmer, India). The flasks were dried in a preheated hot air oven (Memmert Bionixs, India) at 100 °C for 1 h to remove the moisture content and cooled in a desiccator for 1 h again. The content was weighed until two consecutive readings were observed. The fat content was calculated as

$$\text{crude fat}\ (\%) = \frac{[(W4 - W3) \times 100]}{(W2 - W1)}$$

where $W1$ = empty thimble weight, $W2$ = thimble + sample weight, and $W3$ = empty round-bottom flask weight with flat surface.

2.6.4. Estimation of Protein Content. Protein content was quantified using Lowry's method.[15] The protein content was calculated by taking readings at 750 nm using a UV-3000 spectrophotometer (Lab India Analytical Instruments Pvt Ltd., India) against the standard curve prepared using bovine serum albumin.

2.6.5. Estimation of Total Dietary Fiber. The dietary fiber content in samples was quantified through the protocol as described previously.[25] For this, 1 g of AP pulp was added in 50 mL of phosphate buffer and dissolved. Then, 150 μL of heat-stable α-amylase was added in the above solution and heated at 95 °C for 30 min. To remove protein and starch, solution was digested with 150 μL of a protease solution (50 mg/mL) and 200 μL of amylo-glucosidase at 60 °C for 30 min. Then, preheated 95% of ethanol was added to precipitate the solution. The obtained solution was filtered using a VELP Scientifica filtration unit. The retained fibers were dried in oven at 105 °C and weighed for further calculations. For weight correction, protein and ash were identified and subtracted from fiber residue.

2.6.6. Estimation of Carbohydrates. The carbohydrate content was calculated by the formula as described below[24]

$$\text{carbohydrate}\ (\%) = 100 - (\text{moisture} + \text{ash} + \text{fat} + \text{protein} + \text{fibre})$$

2.6.7. Estimation of Total Phenolic Content (TPC). TPC was calculated using the Folin−Ciocalteau method.[24] AP powder (2 g) was extracted using 80% aqueous methanol solution and homogenized at 125 rpm, for 16 h. The suspension was centrifuged at 10,000 rpm for 15 min. To 100 μL of extract, 10-fold diluted Folin−Ciocalteau reagent (1.5 mL) was added and incubated for 10 min. Next, 7.5% sodium carbonate was added, vortex, and after 60 min incubation, the absorbance was measured at 764 nm. The results were presented as mg gallic acid equivalent per 100 g of the sample.

2.6.8. Determination of Antioxidant Activity by DPPH Method. The ability of AP powder to scavenge the DPPH radicals was evaluated using the previous methods.[24] 20 μL of methanol extract was mixed with 3.9 mL of methanolic DPPH (60 μmol/L) solution and kept in the dark for 30 min at room temperature. DPPH solution prepared in methanol was used as a control, while 100% methanol was used as a blank and the absorbance was taken at 517 nm. The results were calculated as inhibition percentage and following formula was implied

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$$\text{inhibition (\%)} = [(\text{absorbance of control} - \text{absorbance of sample})/\text{absorbance of control}] \times 100$$

2.7. Animal Study. The Institutional Animal Ethics Committee of NABI (NABI/2039/CPCSEA/IAEC/2020/11) approved the study and carried out as per the guidelines or the committee for control and supervision on experiments on animals (CPCSEA). Female Sprague-Dawley (SD) rats weighing 150−200 g and aged 6−8 weeks were procured from the Central Animal Facility of the Institute of Microbial Technology in Chandigarh, Punjab, and subsequently housed in the Animal Experimentation Facility of the National Agri-Food Biotechnology Institute (NABI) in Mohali, Punjab. The rats were housed in normal cages with free food and water access, a 12 h light/dark cycle, a constant temperature of 25 ± 1 °C, and a relative humidity of 60 ± 5%.

2.8. Animal Grouping and Experimental Design. Before the experiment, all animals were acclimated to the laboratory surroundings for 1 week and were supplied with normal pellet diet which was purchased from Hylasco Biotechnology Pvt. Ltd., India. Rats were randomly assigned into four groups (n = 6): normal pellet diet control (NPD), 0.5% AP, 1.5% AP and 3% AP. According to food and drug administration's (FDA's) Redbook 2000:IV.C.3.a, Toxicological principles for the safety assessment of food ingredients, for short-term toxicity studies, a duration of 30 days or less is required. Therefore, 4 week study was plan in order to estimate the toxicity of AP on rats.[14] Each group was treated with different concentrations of AP for 4 weeks, as shown in Table 1. During the entire experiment, body weights, food, and water consumption by the rats were measured each week. At the end of study, blood samples were collected and serum was separated and were immediately stored at −80 °C for further biochemical assays. After blood sample collection, all the rats were sacrificed. The liver, kidney, and colon were removed, washed in PBS (phosphate buffer saline), and measured for the organ index. Sections of each organ were then fixed in 10% formalin (pH 6.9) for biochemical and histological examinations.

Table 1. Diet Composition in Grams

diet (% age per 100 g)	composition (%) of normal diet (control)	0.5% AP	1.5% AP	3% AP
carbohydrates	55.90	55.90	55.90	55.90
protein	18.40	18.40	18.40	18.40
fat	05.70	05.70	05.70	05.70
fiber	12.30	12.30	12.30	12.30
moisture	08.00	08.00	08.00	08.00
AP	0.00	00.50	01.50	03.00

2.9. Oral Glucose Tolerance Test. Oral glucose tolerance test (OGTT) was performed after 4 weeks of sample administration. Before initiating the OGTT, animals were kept on fasting for 12 h. Then, the rats were orally administered with glucose (2 g/kg b.w.), and blood samples were collected through tail snipping method at different time intervals (0, 15, 30, 60, and 120 min) after glucose administration to measure blood glucose levels. The amount of glucose response was represented by the area under the curve (AUC) over 120 min for the OGTT data. GraphPad Prism, version 5.0, was used to calculate the AUC.

2.10. Biochemical Analysis. Total cholesterol (TC), low density lipoprotein (LDL), high density lipoprotein (HDL), triglycerides (TG), creatinine, urea, and C-reactive content (CRP) content were all determined by colorimetric method using commercial kits through an automatic biochemical analyzer (Hitachi, Japan).

2.11. Histological Evaluation. Tissue sections of the liver, kidney, and colon were fixed in paraffin wax and preserved with 10% formalin for histological investigation. Hematoxylin−eosin-stained sections (5 mm in thickness) were examined using a compound microscope (Leica, India; 20× magnification).

2.12. Statistical Analyses. The data were represented as mean ± SD. The data were analyzed statistically using GraphPad Prism version 5.0. Unpaired two-tail t-test was used to determine the significance level for chemical analysis, TPC, and antioxidant activity. For food consumption and water intake, Two-way ANOVA was used, while one-way ANOVA was performed for color analysis, body weight changes, OGTT, liver-kidney index, and biochemical analysis followed by Dunnett's test.

3. RESULTS

3.1. Screening of Antibrowning Agents. A number of trials gave little to no changes in the color of AP after drying in a vacuum oven. The original AP (control) showed brown color. Treatment with sodium hypochlorite (4 ppm) and hydrogen peroxide (1%) showed a gray appearance. Oxalic acid (1%), acetic acid (1%), and sodium hexametaphosphate (1%), however, did not exhibit any change in color, whereas citric acid (1%), sodium hexametaphosphate (1%), and oxalic acid (1%), showed slight differences in the color change due to reversible process for debrowning, which means the *ortho*-quinones were not completely converted into ortho-phenols or if converted then reversed again into quinones. Additionally, L-ascorbic acid and L-cysteine had the opposite effect on the debrowning of AP when combined with phosphoric acid (1%), citric acid (1%), and 1% L-ascorbic acid. Following visual inspection, it was determined that L-ascorbic acid and L-cysteine significantly decreased the initiation of oxidation in AP and were consequently optimized using RSM.

3.2. Response Surface Methodology for Antibrowning of AP: Model Fitting and Optimization Conditions. The debrowning of AP was done through RSM. The effect of different concentration of antibrowning agents (L-ascorbic acid and L-cysteine), at different mixing time (min), temperature (°C), and rotation per minute (rpm) was observed against the two responses, i.e., yield (%) and color (L^*-value) and consequently, 2FI model and a linear model was suggested, respectively. The relationship between the variables and responses is stated by the final equation in terms of coded factors as

$$\begin{aligned}\text{yield \%} = {} & 56.35 + 3.71A + 1.19B - 0.5420C + 1.19D \\ & - 0.6208E - 0.0250\text{AB} - 2.10\text{AC} + 1.41\text{AD} \\ & + 0.4525\text{AE} + 2.69\text{BC} - 1.25\text{BD} - 0.2925\text{BE} \\ & - 2.90\text{CD} - 2.90\text{CE} + 2.64\text{DE}\end{aligned}$$

$$\begin{aligned}\text{colour } (L^*\text{-value}) = {} & 54.41 + 2.20A + 3.98B + 0.9186C \\ & - 0.6583D + 0.3000E\end{aligned}$$

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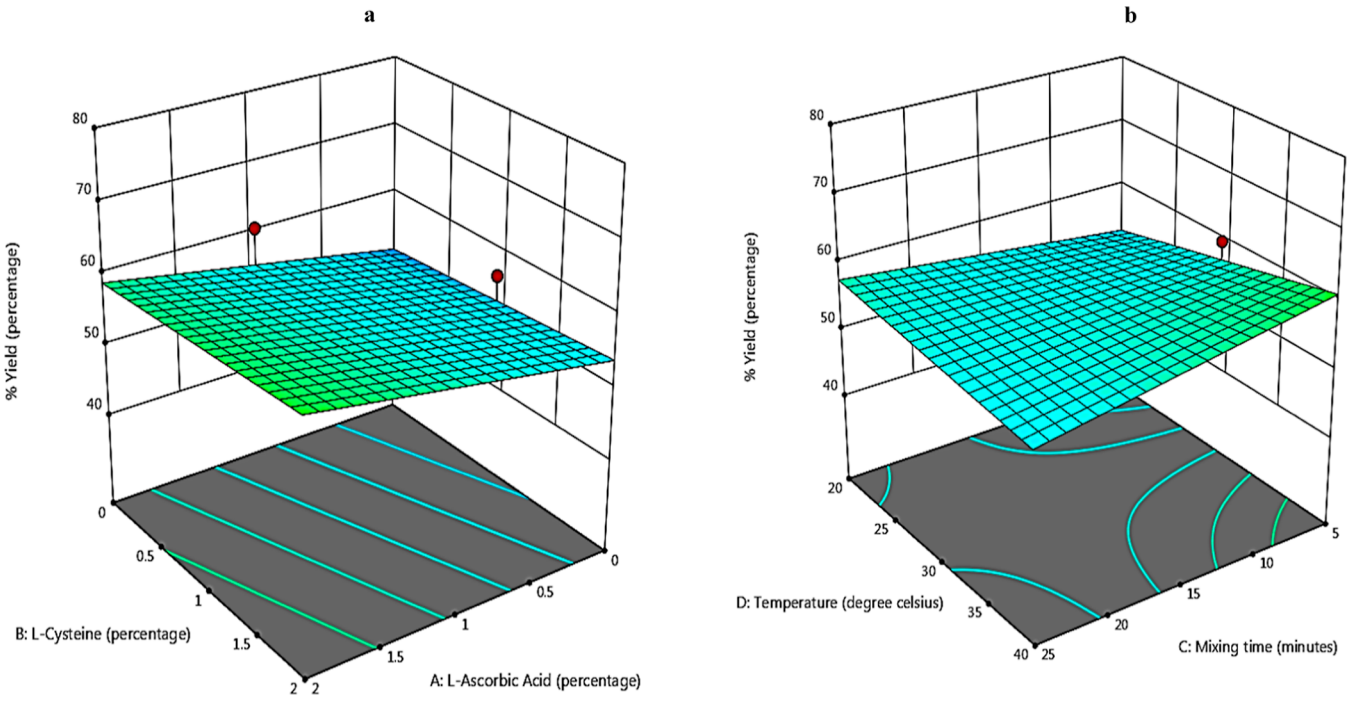

Figure 1. Response surface graph for yield % of AP; (a) L-ascorbic acid & L-cysteine; and (b) temperature and mixing time.

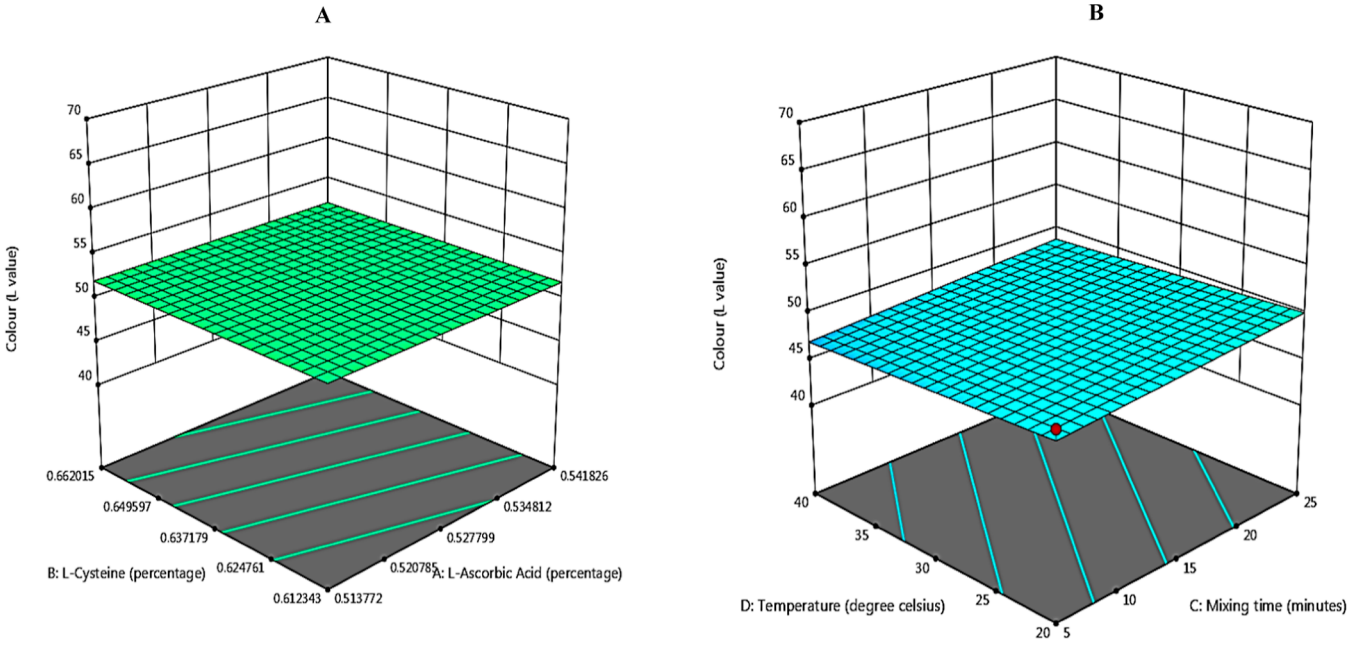

Figure 2. Response surface graph for color (L^*-value) of AP; (a) L-ascorbic acid and L-cysteine; and (b) temperature and mixing time.

where 56.35 and 54.41 are the intercept value of the yield (%) and color (L^*-value), respectively. *A*, *B*, *C*, *D*, and *E* are L-ascorbic, L-cysteine, mixing time, temperature, and rpm, respectively.

Both responses of AP (yield and color) had a significant F-value and a nonsignificant lack of fit ($p > 0.05$). Therefore, the model fitted was significant and suitable for measuring both responses of AP. The relation of coefficient of determination, i.e., R^2 and adjusted R^2 values for both yield (%) and color (L^*-value) were observed in close agreement by the models. The effects of independent variables on the response, yield (%), and color (L^*-value) of AP are given by plotting the 3D surface graphs in Figures 1 and 2, respectively, by taking any one factor constant against any other two independent factors.

Therefore, it may be concluded that with the increase in the concentration of L-ascorbic acid (w/v) and L-cysteine (w/v), pomace yield (%) increased as shown in Figure 1a and also had a significant effect on the L^*-value of AP (Figure 2a). In comparison, mixing time, temperature, and rpm had a less significant effect on yield (%) of AP (Figures 1 and S1a,b) (Supporting Information) and had a nonsignificant or linear effect on the color (L^*-value) of AP (Figures 2b and S2a,b) (Supporting Information). Thus, L-ascorbic acid was the primary determining factor for both yield as well as color of

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AP and L-cysteine can be considered as the secondary determining factor. Based on these observations and the significance of the model, the optimized conditions for yield and color of AP were obtained at 1% concentration (w/v) of L-ascorbic acid and L-cysteine, 15 min mixing time, 30 °C temperature, and 300 rpm.

3.3. Color Analysis and Water Holding Capacity of Treated AP. As shown in Table 2, treated AP has greater L^*-value ($p < 0.05$) in comparison to the control, confirming a favorable impact of antibrowning agents on the color parameters. In addition, the WHC of treated AP ($p < 0.0005$) was significantly higher as compared to control AP (Figure 3a).

Table 2. Color Analysis Representing CIE L^*, a^*, and b^* Space of Control and Treated AP

sample	L^*-value	a^*-value	b^*-value
control AP	46 ± 0.7[b]	9.7 ± 0.6[a]	19.6 ± 0.7[b]
treated AP	68.46 ± 0.8[a]	6.4 ± 0.5[b]	27.4 ± 0.9[a]

3.4. Morphological Analysis of AP. Surface morphologies of control and treated AP was observed by SEM and shown in Figure 3. The treated AP had a rougher surface (Figure 3e as compared to control AP Figure 3d). This rough surface in treated AP resulted in a more porous structure of fibers which helped in the absorption of more water content, leading to the higher WHC of pomace.

3.5. Proximate Analysis of AP. Proximate composition of AP is summarized in Table 3. Vacuum drying of AP, led to a lower fat content (3.99%). There was no significant change observed in the moisture content between control and treated AP. However, there was a significant increase in the TDF (49.65%), protein (7.2%), carbohydrate (48.30%), and amelioration in the ash content (1.25%) as compared to control AP.

Table 3. Proximate Data of AP (on Dry Weight Basis)

composition (%)	control AP	treated AP
moisture	7.23 ± 1.27	7.50 ± 1.20
fat	7.99 ± 0.00	3.99 ± 0.00****
protein	3.86 ± 0.31	7.27 ± 0.16****
ash	1.26 ± 0.00	1.68 ± 0.01****
TDF	31.35 ± 2.49	49.65 ± 4.34**
carbohydrates	29.9 ± 0.58	48.30 ± 0.59***

3.6. TPC and Antioxidant Activity. As demonstrated in Figure 3b, the phenolic content of treated AP has a higher TPC concentration than control AP ($p < 0.0001$). In addition, the antioxidant activity of treated AP was significantly higher ($p = 0.01$) than control AP, as shown in Figure 3c. In addition, 37% inhibition was measured by the methanolic extract of treated AP against control 32%.

3.7. Glucose Homeostasis Effect of AP In Vivo. Figure 4 shows the OGTT results. The blood glucose in all groups reached its peak value in 30 min and that in treated and control group reached to almost its initial level gradually. The AUC could not indicate the potent change in the blood glucose tolerance between the groups treated with AP and the control group.

3.8. Effect of Treated AP on Body Weight, Organ Index, Food, and Water Intake. In this study, no significant change in body weight was found in the groups fed with treated AP and control group (Figure 5a) The administration of treated AP did not alter the organ weight (Figure 5b–d). The weight of the liver, kidney index was not altered after the consumption of treated AP. The food and water intake

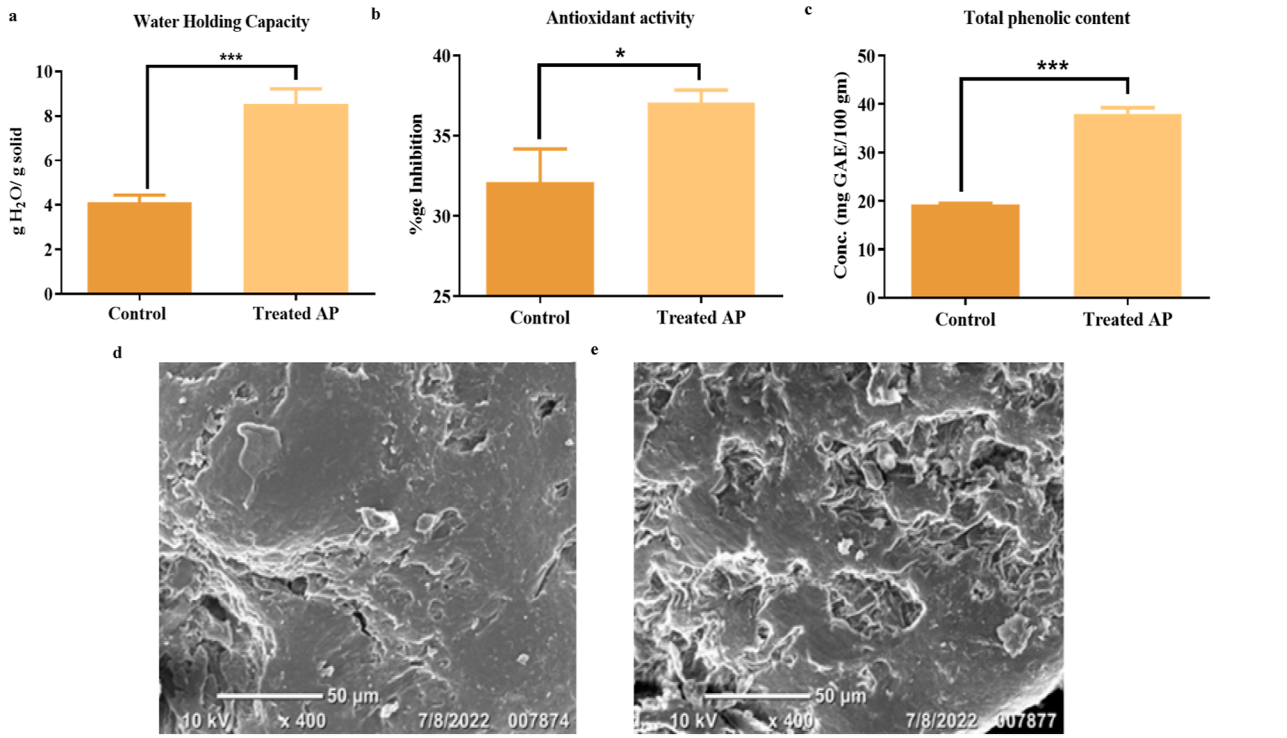

Figure 3. (a) WHC (g H_2O/g solid), (b) antioxidant activity, (c) TPC, (d) SEM images of control AP, and (e) SEM images of treated AP *Values that have different superscripts are significantly different ($p < 0.0001$) according to the unpaired two-tailed t-test at the 95% confidence level.

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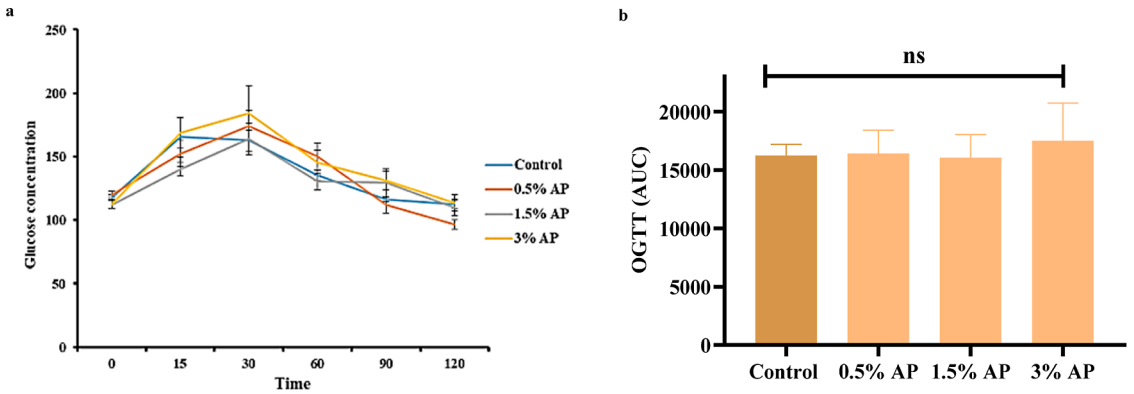

Figure 4. Effect of AP on glucose homeostasis; (a) OGTT and (b) AUC analysis for glucose tolerance test. All values are expressed as mean ± SD. Statistical analysis was done using one-way ANOVA. * ns indicates nonsignificant change.

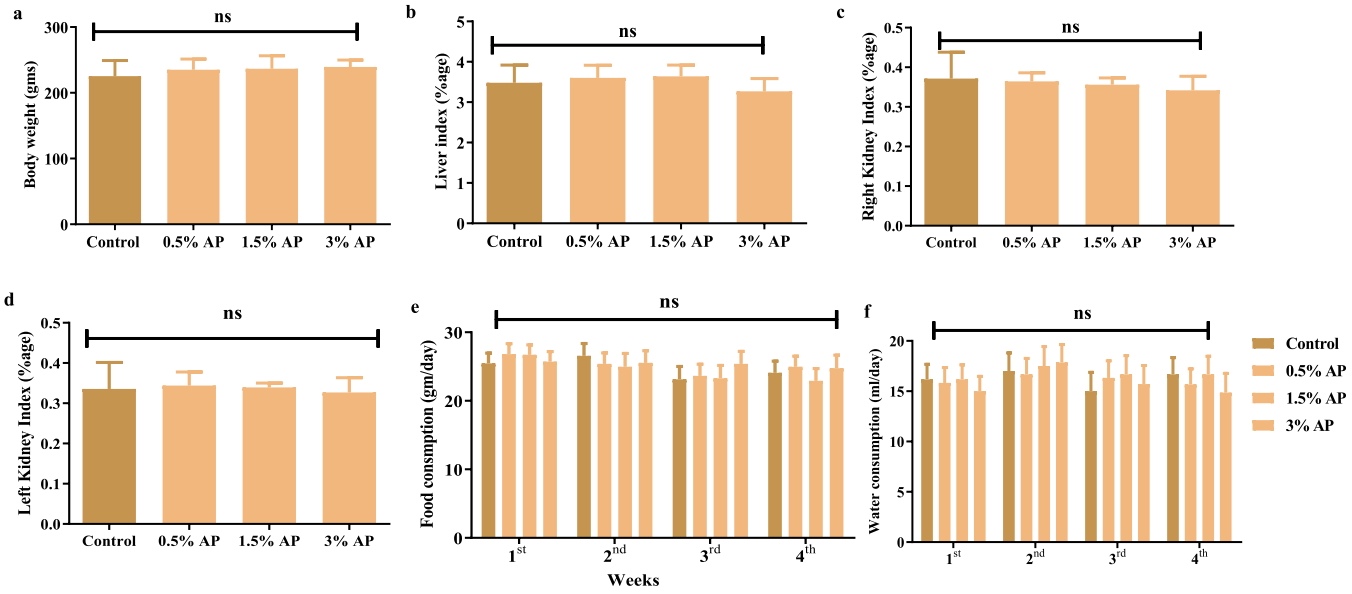

Figure 5. Effect of AP on (a) body weight, (b) liver index, (c) left kidney index, (d) right kidney index, (e) food consumption, and (f) water consumption. All values are expressed as mean ± SD. Statistical analysis was done using one-way ANOVA. * ns indicates nonsignificant change.

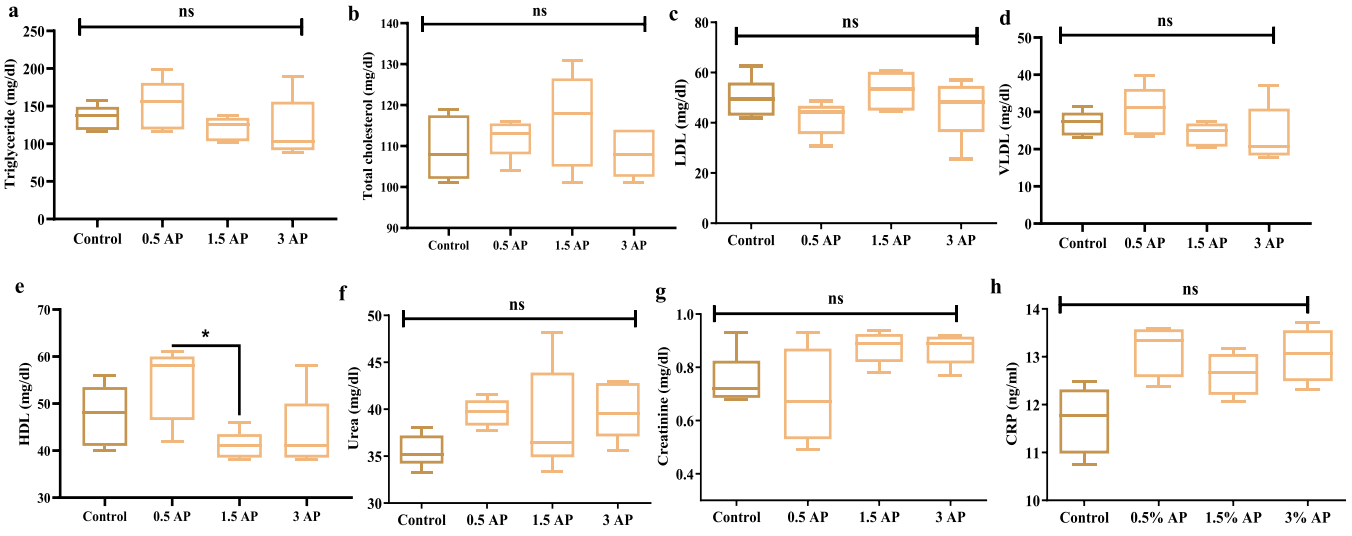

Figure 6. Effect of AP on lipid profile, urea, creatinine, and CRP content; (A) triglycerides, (B) HDL, (C) VLDL, (D) LDL, (E) cholesterol, (F) urea, (G) creatinine, and (H) CRP. All values are expressed as mean ± SD. Statistical analysis was done using one-way ANOVA, * ns indicates nonsignificant change. Abbreviations: HDL: high density lipoprotein, VLDL: very low density lipoprotein, and LDL: low density lipoprotein.

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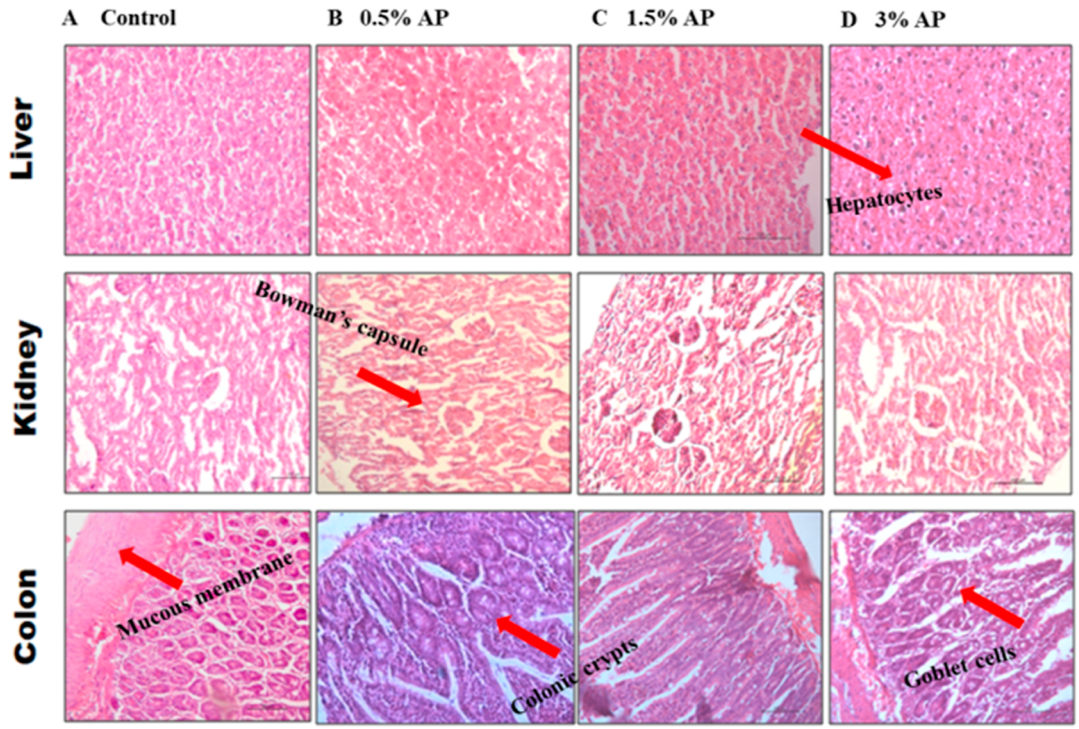

Figure 7. Representative hematoxylin/eosin (H & E)-stained liver, kidney, and colon tissues; (a) control, (b) 0.5% AP, (c) 1.5% AP, and (d) 3% AP, *H & E staining; original magnification at 20×, abbreviation: AP: apple pomace.

between the control and treated group was negligible over 4 weeks (Figure 5e,f).

3.9. Effect of Treated AP on Serum Lipid Profile, Urea, Creatinine, and CRP Levels. The effects of treated AP on lipid levels are summarized in Figure 6a−e. Compared with normal control, there was no significant increase in the levels of TC, triglycerides, HDL, and LDL. Similarly, the serum urea and creatinine levels were not altered in the groups fed with treated groups as compared to the control group, as shown in Figure 6f,g. In addition, CRP levels were maintained in all the groups (Figure 6h).

3.10. Histopathological Assay. The liver histology results showed usual hepatic cell morphology, compared with the normal control group, and no severe liver injuries such as infiltration of lymphocytes and focal necrosis were observed in the liver. Hepatocytic cells were intact with round centrally placed nuclei. Apart from that, the histopathology of the kidney tissue showed normal architecture and no glomerular hypertrophy, sclerosis found in the groups treated with AP. Similarly, the colon tissue remained unchanged and no associated changes were found in the muscularis mucosa, submucosa layer, goblet cells, colonic crypts, and columnar absorptive cells (Figure 7).

3.11. Discussion. Enzymatic browning is regarded as one of the important natural events that occur during food preparation, storage, transportation, and harvesting, affecting the caliber of numerous items and customer acceptability. High molecular weight insoluble polyphenols precipitate on apple cell walls during apple peeling, cutting, slicing, dicing, grinding, and juice extraction. Different fruits and vegetables, such as apples and their byproducts, turn brown as a result of the endogenous polyphenol oxidase enzyme oxidizing these intracellular phenols.[12,19] Antibrowning compounds can be used singly or in combination to prevent the enzyme PPO (polyphenol oxidase) from oxidizing phenols. This sustainable approach aligns with the broader goal of minimizing food waste and promoting environmentally conscious practices in the food industry, particularly concerning agri-food waste.[12]

Antioxidants either convert enzymatically generated endogenic *ortho*-quinones into colorless diphenols or irreversibly react with them to form stable colorless products by limiting oxygen or interacting with intermediary products, breaking the chain reaction and preventing brown pigment. Ascorbic acid, hexylresorcinol, erythorbic acid, glutathione, and cysteine have been examined to inhibit browning.[12,26,27] Ascorbic acid, the most extensively used antioxidant, converts *ortho*-quinones into diphenols, preventing browning.[16,17] Ascorbic acid is a competitive inhibitor and PPO can directly oxidize it. Dehydroascorbic acid was irreversibly oxidized during the reduction process. Ascorbic acid's ability to reduce quinones is restricted by its consumption during browning. Thus, ascorbic acid has been tested in conjunction with various antibrowning compounds, and in this investigation, 15 min was the optimal period to suppress PPO browning.[12]

Cysteine in greater quantities (>1.0%) reacted with the quinone to produce colorless end-products, but at lower concentrations, it served as a competitive inhibitor.[16] In this work, L-ascorbic acid and L-cysteine increased L^*-values and decreased AP browning. Following sight observations in the color of AP, it was determined that L-ascorbic acid and L-cysteine significantly decreased the initiation of oxidation in AP. It can be attributed that L-ascorbic acid may have reduced the oxidized substrate and product *ortho*-quinones back to diphenols.[16,18] On the other hand, L-cysteine is not only a reducing agent but is also a quinone coupler and a chelator, where along with L-ascorbic acid, the pastry dough color improved.[28] Therefore, both L-ascorbic acid and L-cysteine were consequently optimized using RSM. Additionally, PPO's ideal pH is 6−7; therefore, antibrowning chemicals like L-ascorbic acid and L-cysteine are used to keep AP's pH below 4

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to preserve or decrease catalytic activity. Temperature and rpm did not affect AP color.

The dehydration of AP is a promising approach to extend its shelf life, reducing cost during handling and transportation, and facilitating proper storage by creating more space.[8,29] Vacuum drying is an efficient and cost-effective process, as it offers rapid drying under reduced pressure and oxygen deficient environment, improving the shelf life and nutritive quality of the product.[20,30,31] A continuous vacuum-belt drier dried AP in 1.6−2.83 h in an oxygen-deficient atmosphere, preserving its nutritional and functional qualities and preventing oxidation.[20] According to a report,[30] 10% moisture was found in vacuum-dried AP and found 9% moisture after freeze-drying.[15] In our investigation, vacuum drying AP reduced moisture content without changing control or treated AP. Thus, vacuum drying retained less moisture and yielded AP with acceptable biological characteristics.

L-ascorbic acid, an antibrowning agent, and an antioxidant, may explain the high phenolic concentration in treated AP powder. The temperature sensitive polyphenols such as flavonoids, phenolic acid, may be preserved by pretreatments using additives. Certain research indicates that modifications in the structure of tissues can release bound polyphenols, resulting in elevated antioxidant activity.[32] The radical scavenging capability of treated AP depends on phenols and L-ascorbic acid, which is utilized to prevent AP from browning.[32] As the high phenolic content is good for human health,[33] we wanted to compare the phenolic content of the AP with treated AP. The phenolic profile of samples revealed that TPC of treated AP was higher than control non treated AP. The treated AP had shown better antioxidant activity compared to control with a percent inhibition of 37%. AP was treated with ascorbic acid and cysteine in combination for the debrowning of AP. It stops the polyphenol oxidase activity, and thus, if there is a formation of a more stable end product, i.e., orthophenols, therefore, an increase in antioxidant activity was observed.

Dried and crushed AP powder reduces off-odors, transportation, and storage costs, extending its lifespan. Oxygen, enzymes, and drying affect AP color.[21] The color index of treated AP using comparable procedures to the current investigation is scarce. However, Hunter Color CIE L^*, a^*, and b^*-values for raw AP were 33.8, 13.73, and 29.2, indicating a darker look.[21] Another research found decreased lightness values in vacuum-dried AP at 80−110 °C.[20] The L^* value of treated AP was 68.46, indicating that enzyme browning has decreased significantly compared to control AP, which was 46. As previously mentioned, PPO antibrowning chemicals boost the lightness value. AP color also depends on the apple cultivar used to make apple juice, squash, concentrates, or vinegar. SEM was utilized to observe the surface structure of AP, revealing a round and porous configuration.[34] The present study affirms that untreated AP exhibits more tightly arranged and distinct fiber layers compared to the treated AP. SEM images show that treated AP's rough surface caused fibers to absorb more water, increasing pomace's WHC.[5] AP is rich in carbs, fiber, ash, proteins, vitamins, and minerals.[35] Compared to control AP, we discovered substantial increases in TDF, protein, ash, and reduction in fat. The combining effects of pretreatment, have shown improvement in the dietary fiber content.[35] According to a study., higher WHC is linked to higher dietary fiber, ash, and protein, and it further enhances the viscosity of the food. Higher WHC helps to increase the stool weight and potentially slows the rate of nutrient absorption from the intestine.[36] In addition, the constituents vary due to the treatment and the treated AP matrix also binds with debrowning agents, which leads to an increase in the weight of dietary fibers compared to the control. AP may have excellent water retention capacity due to abundant dietary fibers.[8,37] Fiber reduces the risk of cardiovascular disease, obesity, high blood pressure, diabetes, and gastrointestinal health. Dietary fiber decreases serum cholesterol, weight loss, blood glucose levels, and immune response.[38]

In a study, it was observed that adding AP to the pellet diet did not influence food consumption or weight growth, even with increased fiber.[39] AP did not cause clinical symptoms, harmful consequences, or animal fatalities. According to one study,[40] no significant change in organ weight or mortality was observed, and both the test and control groups showed similar postprandial glycemic responses.

This is important for diabetics who must control blood glucose levels to prevent pancreatic beta cells, postprandial hyperinsulinemia, insulin resistance, obesity, and atherosclerosis.[35] Dietary fiber may reduce blood lipid levels by aiding carbohydrate digestion and absorption.[35] Changing life style and food habits cause asymptomatic elevation of various inflammatory markers such as C-reactive protein (CRP).[41] However, since oxidative stress is regarded as a mediator of both acute and long-term liver injury, we were unable to detect variations in the levels of CRP in our study. Also, AP dietary fiber did not affect blood cholesterol, urea, or creatinine levels in the control and treatment groups, although this can be confirmed by using higher concentration of dietary fiber in future experiments. Another study reported that[40] the apple polyphenol extract did not influence tissue histopathology. We found no liver macrophage infiltration or hepatocyte necrosis. Hepatocytic cells have complete circular central nuclei. Bowman capsule was unaltered in the renal tissue. Lymphocytic cells were absent. The muscularis mucosa, submucosa layer, goblet cells, intestinal crypts, and columnar absorptive cells also remained unaltered. So, this finding suggested that the consumption of AP dietary fibers in growing rats did not significantly alter their usual growth, lipid levels, and shows no toxic effect on tissues with the increase in the concentration of AP.

3.12. Conclusions. Pomace generated during the apple juice processing can be managed and preserved successfully, preventing its browning and allowing for its utilization as a sustainable food substitute or in food fortification. This approach aligns with the goal of maximizing resource use and minimizing waste, contributing to a more sustainable and environmentally friendly bioeconomy. In a screening of antibrowning agents, L-ascorbic acid and L-cysteine were shown to work well together, and this combination was further optimized using RSM, with the best results found in the central spots. The treatment with the combination produced higher yield and effective color changes were observed in the AP. In addition, the nutritional composition of treated AP was explored, and the result showed the better physiochemical properties such as improved proximate composition, antioxidant profile, total phenolic, and WHC as compared to the untreated vacuum drying AP. SEM also confirmed the greater WHC by displaying more porosity and compact layers of fiber. Moreover, a 4 week acute toxicology study was performed to determine the effect of AP where the female SD rats were fed with various concentrations of AP. Results demonstrated no

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alterations in the glucose homeostasis, lipid profile, and inflammatory markers. Apart from this, histological studies did not show any morphological changes in the tissues, depicting no toxic effect of treated AP. Thus, present exploration proves that the consumption of AP does not cause any acute toxicity and that this dietary addition of AP is sustainable for consumption, indicating promising longevity and higher sustainability in supporting the well-being of human health.

Furthermore, more preclinical and clinical studies are needed to prove its beneficial effect in treating various metabolic diseases.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c03966.

Response surface graph for % yield of AP: RPM and mixing time, and RPM and temperature; response surface graph for the color of AP: RPM and mixing time, and RPM and temperature; and effect of independent variables on the antibrowning process of AP (PDF)

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Author Contributions

#N.C. and V.T. contributed equally. M.K., M.B. designed the study; N.C. and V.T. carried out the major experiments and analysed the data and drafted the manuscript; A.S., A.K. helped technically in few experiments. M.K., M.G., S.K.K. and A.B. reviewed the manuscript.

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Notes

The authors declare no competing financial interest.

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