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(RESEARCH ARTICLE)

Mixture effects of three analgesics on biochemical indices in rat renal mitochondrial fraction

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Abstract

Humans are exposed to different mixtures of biologically active chemicals, unintentionally or intentionally for example by medications or through foods. The exposure risks of these chemical combinations are far from being understood. According to the World Health Organization, evidence has shown that chemicals without action at individual levels may act additively and cause problems. Thus, human exposure to a mixture could produce biological responses which may not be predictable when used as single compounds. Drug combination is a common intervention in pain management, especially in patients with comorbidities and complex pain syndromes. Aspirin, caffeine and paracetamol combination has been used in pain management but their toxicological implications have not been clearly worked out. The widespread use of analgesics such as paracetamol, aspirin and indomethacin for pain management has raised concerns about their potential adverse effects, particularly on vital organs such as the kidneys. This study aimed to investigate the mixture effect of three commonly prescribed analgesics on the kidney mitochondrial fraction of Sprague Dawley rats. They were orally exposed in 2 mL water to aspirin, paracetamol and indomethacin singly and in combination at their respective therapeutic doses daily for 14 days. The control rats received water and food only. Certain biochemical parameters were assessed in the rats' kidney mitochondrial fraction by spectrophotometric techniques. The statistical analysis of data was done using Graphpad prism software. Data were expressed as Mean ± SEM. The means were subjected to one-way analysis of variance using Tukey as a post-hoc analysis. Values were considered statistically significant at $p<0.05$. The activity of the superoxide dismutase under the Mix (drug combination group) increased significantly when compared with the respective enzyme activity under the individual drugs. Extent of lipid peroxidation, concentrations of non-protein sulphydryl (NPSH) and creatinine were lower in the Mix group relative to the test groups. Aspirin group recorded the least lipid peroxidation and NPSH concentration while indomethacin group the highest. The highest creatinine concentration was recorded among rats dosed paracetamol. The pro-oxidant ability of the drugs and hence renal toxicity appear to be in the order Paracetamol > Indomethacine > Combination dose > Aspirin. Aspirin effect and presumably mechanism of its toxicity may be antagonistic to the activity of the rest of the drugs. These findings indicated that the drug mixture was less pro-oxidant than the individual drugs at their therapeutic doses in the rat kidney.

Keywords: Analgesics; Combination Effects; Oxidative Stress; Mitochondrial Dysfunction; Renal Toxicity

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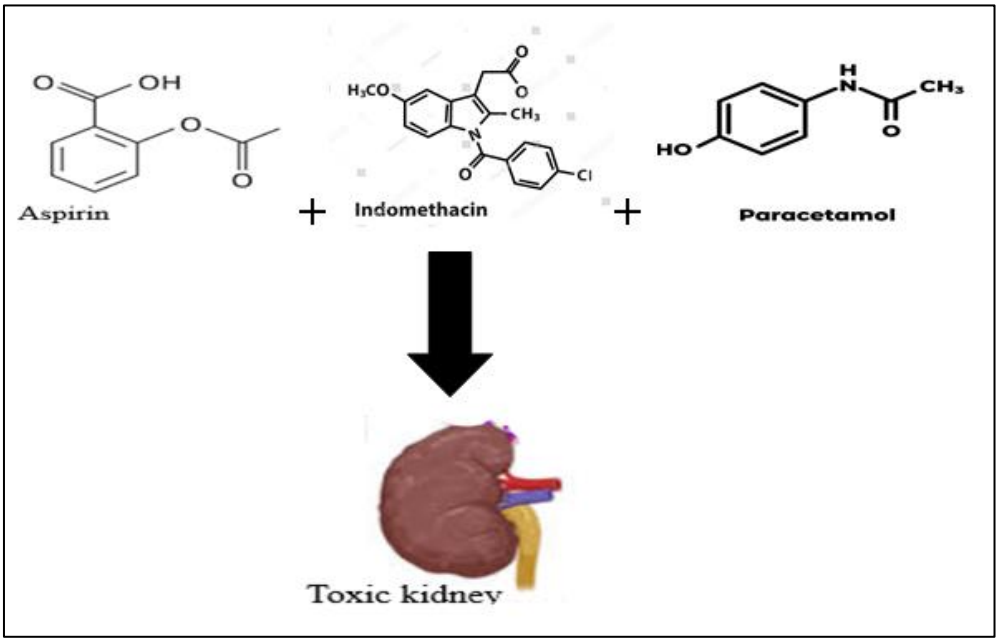
Graphical abstract



1. Introduction

Human and animals are exposed to diverse biologically active chemicals from various environmental media sourced from agriculture, industry, medical facilities, and household wastes Although most anthropogenic compounds in the environment are always present at very low concentrations, "intentional" exposure to these compounds such as by medication [1] occurs at higher concentrations and can as well lead to bioaccumulation in the fatty tissues engendering toxicity [2, 3]. Unintentional human exposure to pollutants in the environmental matrices (air, soil and water) involves complex chemical mixtures of compounds of diverse nature. The toxicological effects of compounds mixture can result in either independence (response additivity) or dose addition. Concentration (or dose) addition has been used in association with mixtures of similar acting chemicals such as those interacting with ER, while independent action has been employed for different acting compounds, including compounds in a mixture that interact with dissimilar receptors or other molecular targets. Thus, human exposure to a mixture could produce biological responses which may not be predictable when using single compounds [4]. According to the World Health Organization [5], there is emerging evidence that many chemicals may act additively and, each at levels without individual effect, could act together to cause health problems.

Pain is a common symptom that can significantly impair an individual's quality of life and productivity. As a result, the use of analgesics, including non-steroidal anti-inflammatory drugs (NSAIDs) and the opioids, has become a standard practice for pain management. Drug combination is a common intervention in pain management, especially in patients with comorbidities and complex pain syndromes [6]. Dose combination of acetylsalicyclic acid (aspirin), paracetamol and caffeine is commonly used mix analgesic [7]. However, the widespread use of analgesics has raised concerns about their potential adverse effects, particularly on vital organs such as the kidneys [8]. Any drug-induced damage to the kidneys can lead to significant health complications, including acute and chronic renal failure. Mitochondria are the cellular organelles responsible for energy production through oxidative phosphorylation. Mitochondrial dysfunction is a key contributor to several pathological conditions, including neurodegenerative disorders, cancer, and cardiovascular diseases. The kidney is a highly energy-demanding organ that relies heavily on mitochondrial function to maintain its physiological functions [9]. Consequently, drug-induced mitochondrial dysfunction in the kidney can lead to renal injury and dysfunction. However, the potential adverse effects of drug combinations on mitochondrial function in the kidney remain largely unexplored. Therefore, understanding the effects of analgesic drug combinations on renal mitochondrial function is critical for the development of safer pain management strategies. In this study, we aimed to decipher the mixture effect of three commonly prescribed analgesics on the kidney mitochondrial fraction of Sprague rats. The selected analgesics included indomethacin a non-steroidal anti-inflammatory drug (NSAID) and mechanistically a non-selective cyclooxygenase (COX) inhibitor [10]. Indomethacin is a derivative of indoleacetic acid and iodoacetic acid. Unlike indomethacin, paracetamol or acetaminophen is not a member of NSAID but is highly selective for cyclooxygenase-2 with a weak anti-inflammatory activity especially in the central nervous system. It is frequently

prescribed with or without combination for pain management particularly in patients with chronic pain syndromes [1,11]. Paracetamol efficacy and safety may be additive or synergistic when prescribed in combination with NSAIDs [12]. Aspirin (Acetylsalicylic acid) is a NSAID and widely used as an analgesic, anti-inflammatory and anti-platelet drug [13]. All the three medications are known inhibitors of prostaglandin synthesis via cyclooxygenase inhibition. Their anti-androgenicity has also been reported in rat foetal testis [14]. To assess the mixture effect of these analgesics, we evaluated key parameters associated with mitochondrial function and integrity and reactive oxygen species (ROS) generation. We hypothesized that the simultaneous administration of the three analgesics at therapeutic doses would result in a synergistic effect leading to mitochondrial dysfunction and oxidative stress within the kidney. The results of this study may have significant implications for the safe use of analgesics in pain management, particularly in patients with renal comorbidities.

2. Materials and methods

2.1. Reagents

All reagents used for this project work were of analytical grade obtained either from Sigma Aldrich Company, Louis USA or from our accredited contractors.

2.2. Experimental design

Apparently healthy adult male Sprague-Dawley rats (average weight 150 ± 3.30 g) were housed under standard conditions of temperature (25ºC ± 2ºC) and humidity (55% ± 10%) with a 12-hour light/dark cycle and fed pelleted diet and water *ad libitum*. They were acclimatized for 2 weeks before the experiments. The rats were randomly divided into five groups, each group contained 5 rats. The first group (the control) consisted of rats fed with basal diet not supplemented with the test drugs. The second group was administered therapeutic dose of aspirin equivalent to 16.7 mg/kg b w/day based on human adult weight of 60 kg [15], the third group received indomethacin administered at 150 mg/kg b w/day [16] therapeutic dose for gouty arthritis, while the fourth group got paracetamol at a maximum dose of 4 g/day or 0.067g/kg body weight (b w)/day [17], the fifth group was administered the mix doses of the drugs daily for 14 days. The drug doses were contained in 2 mL distilled water.

2.3. Sample collection

The animals were sacrificed by decapitation on the 16th day of administration. Blood samples were allowed to clot and the resulting sera carefully aspirated without taken up the cells. The kidneys were harvested washed clean of connective tissues with ice-cold 0.15 M KCl and were homogenized in ice-cold 0.25 M sucrose solution buffered with 40 mM Tris.HCl at pH 7.4 or in 0.02 M ice-cold EDTA in some experiments using Teflon-lined homogenizer in all cases. Renal tissue mitochondrial fraction was obtained by the method of Johnson and Lardy [18] in a 0.25 M sucrose medium with 2 mg/ml of albumin.

2.4. Biochemical Assays

Superoxide dismutase activity was determined by using the method of Misra and Fridovich [19] which is based on inhibition of epinephrine autoxidation by superoxide anions at basic pH (10.2). Fresh epinephrine solution was prepared by dissolving 13.7 mg of epinephrine in 250 mL of distilled water. 1 mL of the sample was diluted to 10 mL with distilled water. The kinetics of the enzyme was followed by reading the absorbances at 480 nm at 30 s intervals for a total time of 150 s against blank made up of the reaction mixtures less the sample. The change in absorbance was finally calculated. Molar extinction coefficient of the adrenochrome at 480 nm was taken as 4020 $M^{-1}cm^{-1}$. The activity of the enzyme was defined as the amount of the enzyme that carried out the half inhibition of the autoxidation of epinephrine to adrenochrome under the stated conditions. Estimation of lipid peroxidation in the renal tissue homogenates was conducted by spectrophotometric determination of malondialdehyde (MDA) concentration [20]. To 1 mL of the sample was added 0.5 ml 2.5% v/v HCl followed by 0.5 ml of thiobarbituric acid solution (1% w/v in 50 mM sodium hydroxide). The reaction mixture was heated at 80°C for 30 min, cooled with running water and the chromogen was extracted with 2 mL of butanol. The absorbance of the upper layer was read at 532 nm. The extinction coefficient was taken as 1.56×10^5 $M^{-1}cm^{-1}$.

The sample volume was 0.5 mL. Concentration of non-protein bound sulphydryl groups (NPSH) was estimated as explained by Sedlak and Lindsay [21]. The tissues were homogenized in ice cold 0.02 M disodium EDTA for this investigation. The absorbance of the resulting solution was read at 412 nm within 5 mins of DTNB addition against a blank without the homogenates. Molar extinction coefficient of 2-nitro-5-thiobenzoic acid (TNB) is 14,150 $M^{-1}cm^{-1}$ [22]. Protein concentration was determined by the spectrophotometric method of Bradford [23] in which Coomassie Brilliant

Blue G-250 dye was dissolved in 95% ethanol followed by addition of 85% phosphoric. The absorbance was read at 595 nm. The procedure was as contained in Bioquant kit manual. A calibration curve was constructed from a series of standard albumin concentrations ranging from 100 to 1000 µg/L. Creatinine concentration was determined by Jaffe reaction as alkaline creatinine picrate [24; 25]. Briefly, 1 mL. 0.04 M picric acid was mixed with 1 mL. 0.75 M sodium hydroxide to which 3 mL of the standard creatinine (0.5 mg/mL prepared in 0.1 M HCl) was added. The orange colour was allowed to develop in 20 minutes; the absorbance was read against distilled water on a spectrophotometer at 520 nm. A series of creatinine concentrations was prepared and subjected to the test from which a calibration graph was constructed. The unknown concentration of creatinine from the protein-free serum sample was read off from the standard graph. Succinate dehydrogenase activity was determined according to the continuous spectrophotometric technique of Munujos and co-workers [26]. Change in absorbance over 6 mins at 500 nm was calculated using the extinction coefficient of 19,300 $M^{-1}cm^{-1}$ [27].

2.5. Statistical Analysis

The statistical analysis of data was done using Graphpad prism (version 9). Data were expressed as mean ± SEM and were subjected to one-way analysis of variance (ANOVA) using Tukey- multi- comparison. Difference between means was considered statistically significant at $p < 0.05$.

3. Results

The effect of the drug treatments on the renal tissues superoxide dismutase (SOD) specific activity are depicted in Fig. 1. Generally the SOD activity was significantly ($p < 0.05$) lower in all treated animals than in the untreated control animals. There were significant differences in SOD activity between Mix (mixture of the drugs) and the test groups except the paracetamol group. The Aspirin group exhibited the lowest specific activity of SOD. The malondialdehyde (MDA) concentrations were significantly increased among paracetamol, indomethacin and Mix exposed rat groups relative to the control (Fig. 2). The MDA concentration was highest in Indomethacin group whereas aspirin induced the least quantity. The difference in MDA concentration between aspirin and the control was significant. No statistically significant difference was recorded between paracetamol exposed rats and the Mix rats; the reverse was the case between Indomethacin and Mix rats.

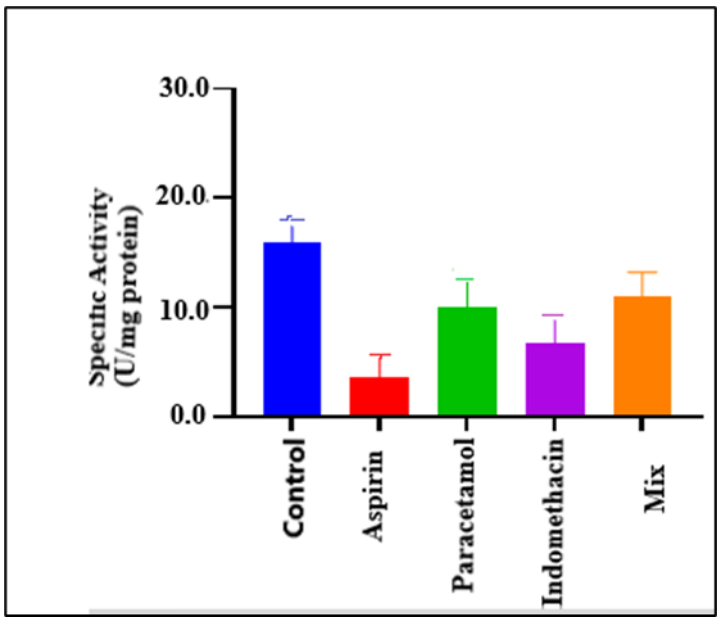


Figure 1 Superoxide dismutase activity in rat renal tissues

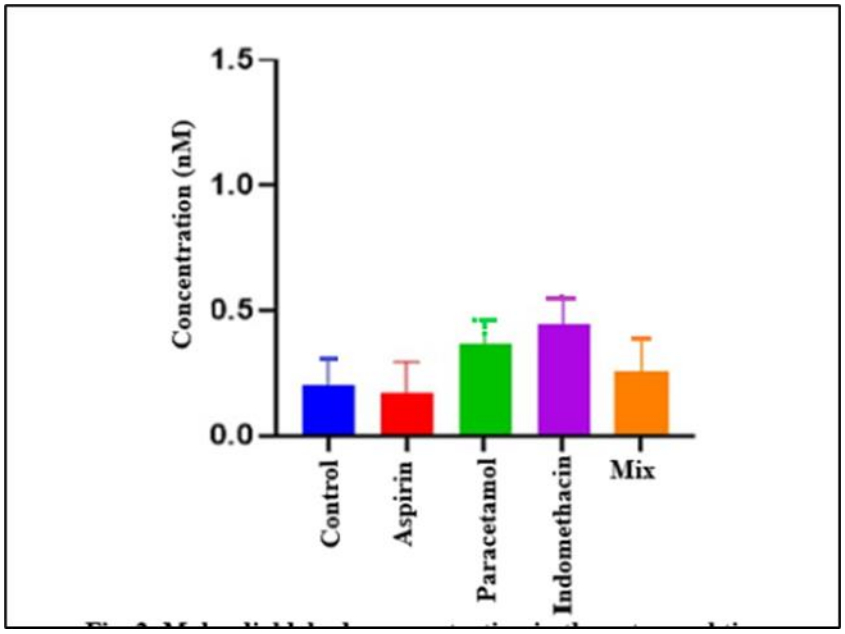


Figure 2 Malondialdehyde concentration in the rats renal tissues

The concentration of non-protein sulphydryl (NPSH) was significantly reduced among all the treated rats when compared with the Control (Fig. 3), where Mix showed the least concentration among the exposed rats. All the exposed groups (aspirin, paracetamol, indomethacin) showed significant increase in NPSH concentration when compared to the Mix (Fig. 3).

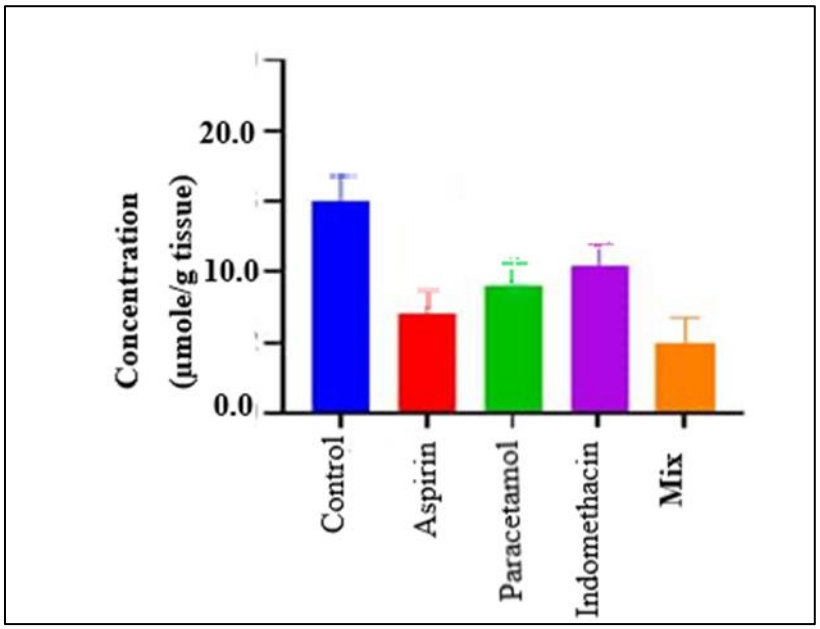


Figure 3 NPSH concentration in the renal tissue of the rats

All the drugs induced significant increase in creatinine concentration compared with the Control (Fig. 4). Paracetamol induced the highest concentration of renal creatinine followed by indomethacin and aspirin in that order. Mix caused the minimal concentration of creatinine among the exposed rats but the differences among the exposed groups were significant.

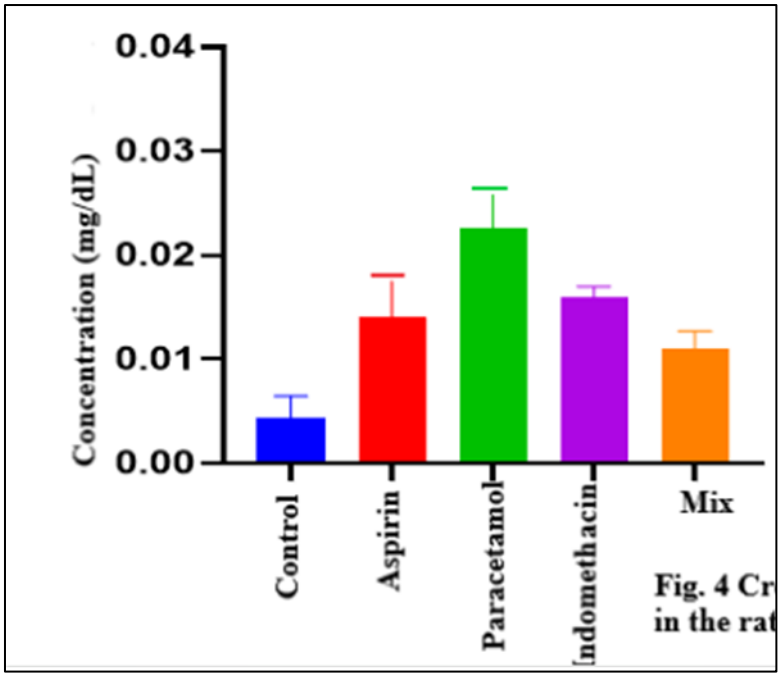


Figure 4 Creatinine concentration in the rat renal tissue

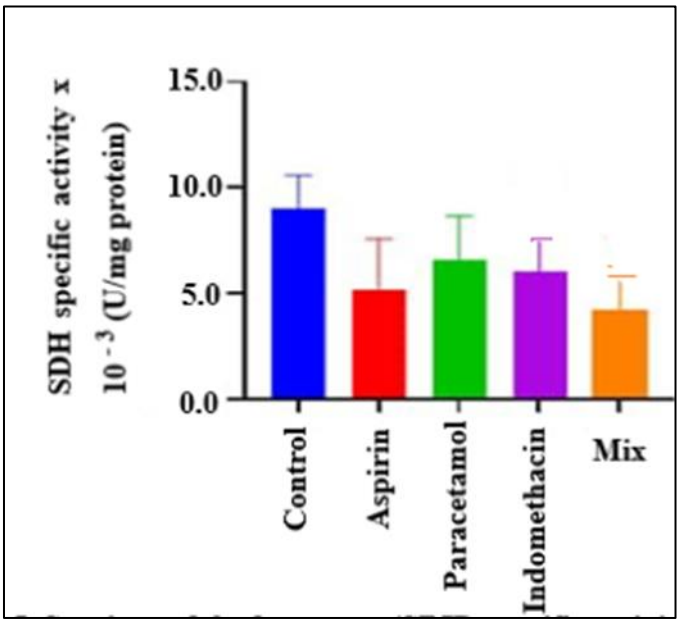


Figure 5 Succinate dehydrogenase (SDH) specific activity in the rats

The activity of SDH was significantly lower in all the exposed rats than the Control (Fig. 5). Each of the component drugs displayed high SDH activity relative to the Mix. Although both paracetamol and indomethacin displayed comparably high SDH activities, the difference was not statistically significant.

4. Discussion

Aside intentional exposure such as in medication, the three drugs can co-exist in environmental media [28, 29, 30] and in contaminated food items [31] through which humans can be inadvertently exposed to the mixtures [32]. The study was accordingly embarked upon to understand the drug mixture toxicity and associated mechanism in the kidney. A study in goldfish model has demonstrated that mixture effects are characterized by a stress response that cannot be predicted from exposure to individual compounds [33]. Reactive oxygen species (ROS) are highly generated in the

mitochondria as a consequence of oxidative phosphorylation. Excessive amount of the free radicals are generated under pathological conditions, affecting redox homeostasis and leading to cellular and tissue damage. Superoxide dismutase is a group of metalloenzymes found in all life forms. The enzyme forms the front line of defence against ROS-mediated injury. The high SOD activity in the control group suggested that the control group had a higher capacity to scavenge free radicals and protect against oxidative damage, which could be attributed to the normal physiological functioning of SOD in maintaining redox balance [34]. The significantly reduced superoxide dismutase activity in the group of rats that received aspirin therefore indicated an alteration of the redox system and an oxidative impact on the kidney that could lead to oxidase stress. The toxicological effect of the combined drugs (Mix) on SOD activity probably illustrated concentration addition or synergy; all the component drugs were presumed to have contributed to produce a greater effect than each of the constituent drugs [35]. Lipid peroxidation, as indicated by MDA concentration is an important marker of oxidative stress and cellular damage. Although there are other aldehydes resulting from lipid peroxidation such as hydroxynonenal, MDA (1, 3-propane dial) is more hydrophilic and by far higher in concentration than other lipid-derived aldehydes. The odd against MDA is that its concentration can be over-estimated because of its similar absorption maximum with other unrelated chemical compounds [36]. The significantly higher MDA concentration in the indomethacin group compared to the paracetamol group suggests a potent pro-oxidant effect of indomethacin in the kidney. The observed reduction of MDA by aspirin could be attributed to its purported activation of antioxidant defence mechanism at low dose [37, 38, 39] and might have contributed significantly to the overall reduction of effect by Mix. Non-protein sulphydryl (NPSH) plays crucial roles in cellular antioxidation and redox homeostasis. NPSH is contributed primarily by reduced glutathione (GSH) and cysteine. Rat kidney is noted for its high concentration of cysteine [40]. The very low concentration of NPSH recorded in Mix group relative to all other groups means renal tissues NPSH was depleted and therefore vulnerable to oxidative damage [41] indicating cumulative effect of the constituent drugs. Succinate dehydrogenase (SDH), a protein assembly in the inner mitochondrial membrane is a member of tricarboxylic acid cycle. The enzyme plays a role in the reduction of ubiquinone to ubiquinol in the mitochondrial electron transport chain. The combination of the drugs produced significantly lower SDH activity. This lowering effect may have resulted from the constituent drugs inability to act cumulatively that would have resulted in additive or synergistic effects [42, 43]. Accordingly, the combination effect on SDH is termed antagonistic [44]. Default in the activity of this enzyme will impact negatively on cellular energy generation and pathogenesis of certain human diseases such as cancers and neurodegeneration [45, 46]. Creatinine is a metabolite of creatine phosphate in the muscle and following its high water solubility is excreted by the kidneys. Serum creatinine is an important marker commonly used to assess muscle mass and kidney function [47]. The observed increase in the serum creatinine among the treated groups could be an indicator of increased loss of muscle mass and renal injury [48]. These results may have lent credence to the previous reports associating paracetamol and indomethacin with nephrotoxicity and renal dysfunction [49]. The average creatinine concentration in the Mix group lower than the sum of the individual drug effects depicted antagonistic effect and is thought of as the constituent drugs operated at different mechanisms of toxicity [44]. Most studies reporting nephrotoxic nature of the drug mixture used very high doses [50, 51] in sharp contrast to this study which used therapeutic doses of the respective drugs.

5. Conclusion

All the drugs and their combination displayed a pro-oxidant activity in the renal tissues at therapeutic doses. The pro-oxidant effect of the combination dose was lower than the individual effects.

Compliance with ethical standards

Disclosure of conflict of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Statement of ethical approval

The study received ethical approval from the LAUTECH Ethical Committee on use of experimental animals.

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