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Therapeutic Efficacy of Silymarin from Nigerian Medicinal Plants in NDEA-Induced Hepatocellular Carcinoma: Impact on NF-κB, IL-6, IL-10, MDA, and LDH

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ABSTRACT: Liver cancer, particularly hepatocellular carcinoma (HCC), is a rising global health concern, driven by risk factors such as HBV, HCV, and NASH. Key biomarkers like malondialdehyde (MDA), lactate dehydrogenase (LDH), NF-kB, and cytokines are indicative of oxidative stress and inflammation associated with HCC progression. Phytochemicals from Nigerian medicinal plants, especially silymarin a potent antioxidant and anti-inflammatory compound derived from milk thistle have shown promising therapeutic potential in mitigating liver damage. These bioactive compound target oxidative damage and immune modulation pathways, offering hope for innovative treatments against HCC. This study investigates the hepatoprotective effects of silymarin on biochemical markers in N-nitrosodiethylamine (NDEA)-induced hepatocellular carcinoma (HCC) in rats, focusing on lactate dehydrogenase (LDH), malondialdehyde (MDA), nuclear factor-kappa B (NF-kB), and cytokine levels. Results indicate that NDEA exposure significantly elevated LDH, MDA, and NF-kB levels in serum and liver tissues, correlating with tissue damage and oxidative stress. Silymarin treatment markedly reduced LDH levels ($p < 0.001$), suggesting their role in mitigating HCC progression. Elevated MDA levels indicated enhanced lipid peroxidation, with silymarin reducing MDA significantly ($p < 0.001$), implicating their antioxidative properties. NF-kB levels, an early marker of neoplastic changes, were notably decreased following silymarin administration, particularly with 75 mg of silymarin, which showed a stronger effect than sorafenib a reference drug. NDEA also increased pro-inflammatory cytokine IL-6, which was dose dependently downregulated by silymarin, whereas IL-10 levels, an anti-inflammatory cytokine, were upregulated in treated groups, aiding in inflammation modulation. This study underscores the therapeutic potential of silymarin, supporting its efficacy in HCC management by reducing oxidative damage, inflammation, and cell proliferation markers

I. INTRODUCTION

Liver cancer remains a significant global health issue, with its incidence steadily rising worldwide [1]. By 2025, it is projected that over 1 million people will be diagnosed with liver cancer annually. Hepatocellular carcinoma (HCC) is the most prevalent form, accounting for approximately 90% of cases. Hepatitis B virus (HBV) infection is the leading risk factor for HCC, responsible for around 50% of cases. The risk posed by the hepatitis C virus (HCV) has notably decreased due to the widespread use of antiviral treatments, leading to sustained virological response (SVR) in many patients. However, individuals with cirrhosis remain at elevated risk for HCC even after HCV clearance. Non-alcoholic steatohepatitis (NASH), linked to metabolic syndrome or diabetes, is emerging as the most rapidly increasing cause of HCC, particularly in Western countries [2].

Hepatocellular carcinoma (HCC) is the most common type of liver cancer and the fourth leading cause of cancer-related deaths globally. It ranks sixth in terms of cancer incidence worldwide and second in total years of life lost due to cancer. The incidence and mortality rates of liver cancer vary significantly by region. The highest age-standardized incidence rates are observed in the Asia-Pacific region, East Asia, and central sub-Saharan Africa. In contrast, the lowest rates are found in southern and tropical Latin America. In countries like Egypt and Thailand, liver cancer is the leading cause of cancer deaths, whereas in Ukraine and Poland, it ranks 14th. The incidence of HCC is notably higher in males [3]. HCC accounts

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for 75–85% of all liver cancers. Globally, liver cancer deaths are mostly due to three risk factors: alcohol use (30%), hepatitis B (33%), and hepatitis C (21%) [4].

Phytochemicals derived from medicinal plants play a crucial role in traditional medicine and have garnered significant attention in recent years due to their therapeutic potential. In Nigeria, a country rich in biodiversity, various indigenous plants are utilized for their medicinal properties, primarily due to their phytochemical constituents. Recent research emphasizes the need for further exploration of the phytochemical constituents of Nigerian medicinal plants [5]. Despite the rich diversity of these plants, many bioactive compounds remain unexplored. For instance, compounds like alloeudesmenol and hanocokinoside have been identified but require more investigation to understand their full therapeutic potential. Additionally, there is a growing interest in the structural optimization of these phytochemicals to develop new drugs that can combat diseases, a significant global health issue. The unique chemical fingerprints of Nigerian medicinal plants could lead to the discovery of novel compounds that address neglected tropical diseases prevalent in the region [6].

A byproduct of polyunsaturated fatty acid peroxidation in cells is the formation of malondialdehyde (MDA). A surge in free radicals results in an excess of MDA generation. The existence of malondialdehyde is commonly cited as evidence of both antioxidant status and oxidative damage in cancer patients. MDA is a chemically active chemical that may interact with proteins' amino acid moieties and DNA, the two aldehyde groups combine with nucleophiles. MDA may produce adducts that damage biomolecules. A prominent approach for measuring the peroxidation of lipids is the thiobarbituric acid reactive substances (TBARS) test [7]. [8] found that NDEA statistically elevated MDA in the liver of NDEA-induced HCC.

Lactate dehydrogenase (LDH) is a specific kind of protein that plays a vital role In the generation of the human body's energy, Body organs such as kidneys, blood, liver, brain, and lungs are just a few of the body tissues where it may be found. LDH escapes into the blood or other body fluids when these organs experience damage. If you have high quantities of LDH in the blood or any physiological fluids, this might suggest that you have sick or wounded tissues [9]. [10] discovered that serum LDH was significantly higher in HCC rats.

Nuclear Factor kappa-B (NF-kB) is an essential transcription factor that controls genes involved in both innate and adaptive immune responses is nuclear factor kappa B (NF-kB). It is essential for controlling several cellular functions, including cell division, survival, and the immunological and inflammatory responses of the body. According to [11], there was an upregulation of NF-kB in the liver of NDEA-induced rats.

Cytokines are small, secreted proteins which involved in a crucial process in cell communication including interaction. They are vital for controlling the development and function of immune system cells as well as blood cells. The release of cytokines signals the immune system to activate its [11]. [12] observed that all blood cells as well as other cells involved in the immunological and inflammatory responses of the body are influenced by cytokines during development. [13] discovered that cancer patients had higher IL-10 concentrations than healthy patients. [14] investigated new treatment drugs for the Pterospermum Lanceifolium (PLE) Roxb leaves' HCC phenolics-rich component against NDEA-induced HCC rat model system and hepatic cancer cell lines. NDEA increased IL-6 in chronic hepatic disorders.

II. MATERIAL AND METHODS

Chemicals

N-nitroso diethylamine with catalog number N525465 and Silymarin were supplied by Carbanio, a chemical firm (India). LDH kit from Agappe Diagnostics (Switzerland). Sorafenib was purchased from Cipla Pharmaceutical Company (India). Kits for the enzyme-linked immunosorbent test (ELISA) of NF-kB, IL-6, and IL-10 with the catalog numbers MB-1731A, MB-1736A, and MB-7455A, in that order. They were purchased from Mornmed Medical Equipment (China). Analytical quality chemicals and reagents were procured from Sigma Aldrich, located in St. Louis, Germany.

Experimental design

Forty-eight (48) male Wistar rats (48) were acclimatized for two weeks before being divided into six groups (n=8). Group 1 served as positive control and received a single intraperitoneal injection (i.p.) of 200 mg/kg of NDEA. Group 2 received an oral dose of 1 ml of corn oil. Group 3 was given an oral dose of 50 mg/kg/bodyweight of silymarin. Group 4 had intraperitoneal (i.p.) NDEA and then 10 mg/kg/bw sorafenib (conventional drug. Groups 5 and 6 were administered NDEA 200 mg/kg/bw and followed

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by silymarin 50 mg/kg and 75 mg/kg, respectively. All treatments occurred for 14 days.

After 6 weeks of administration of N-Nitrosodiethylamine, one rat was sacrificed from each group for gross and histological examination to determine the degree of hepatocellular carcinoma. After histological examination and confirmation of hepatocellular carcinoma, animals were administered orally with daily doses of 50mg/kg, 75mg/kg silymarin, and 10mg/kg sorafenib for 14 days, according to the method of [15].

Blood and Liver Tissue Collection

Animals were fasted for twelve hours before getting their blood samples using the ocular method, after two weeks of treatment.

For hematological examination, 1 milliliter of the experimental animals' blood samples were taken and placed in a plain bottle coated with ethylene diamine tetraacetic acid (EDTA). 15 minutes of centrifugation at 4°C and 2500 rpm was used to separate the sera from the residual blood that had been collected and stored at 4°C for 30 minutes in a plain tube' The kidney and liver were excised immediately, and they were weighed and cleaned with ice-cold normal saline solution. Cut and homogenized 0.2g of liver and kidney were placed in 1.8 milliliters of phosphate buffer (10 mM; pH 7.4). After centrifuging the homogenate, supernatants were transferred into 2 ml Eppendorf tubes after being centrifuged at 4000 rpm for 15 minutes on ice and kept in the freezer for the biochemical test. For gene expression profiling, a tiny part of the liver and kidney were sliced and stored in Eppendorf tubes with 100µl of trizol. A different portion of the kidney and liver were preserved in a 10% phosphate-buffered formalin fixative.

Determination of Lactate Dehydrogenase (LDH) Activity

The diagnostic Agape kit was used to determine lactate dehydrogenase (LDH) by the techniques of [16]; [17]; and [18].

In the process, 10 µL of the sample and 1000 µL of the working reagent were combined, and the mixture was then incubated at 37°C for 1 minute. The change in absorbance per minute (OD/min) was then measured over 3 minutes.

LDH Activity (U/L) = (Δ OD/minutes) x 16030

$$\text{LDH activity (U/mg protein)} = \frac{\text{LDH activity (U/L)}}{\text{Total protein concentration (mg/L)}}$$

Determination of Malondialdehyde (MDA)

The formation of TBARS (thiobarbituric acid reactive substances) was measured using the [19] technique to evaluate the peroxidation of lipids.

A portion of the test sample, equal to 0.4 milliliters, was combined with 0.5 milliliters of 30% TCA and 1.6 milliliters of Tris-KCl buffer. After that, 0.5 ml of 0.75% TBA was added to the sample and heated to 800C for 45 minutes in a water bath. After being chilled in ice to ambient temperature, it underwent a 10-minute, 3000 rpm centrifugation, using distilled water as a reference blank, the absorbance of the obtained clear supernatant was measured at 532nM.

$$\text{nMole MDA / mg protein} = \frac{\text{Absurbance x Vol of mixture}}{\sum_{532} \text{ x Vol of sample Xmg protein}}$$

Measurement of NF-kB, IL-6 and IL-10

This assay makes use of the quantitative sandwich enzyme immunoassay technique. 50µl of the standard was put into a standard well. 10µl of the testing sample and 40µl of sample diluent were added to the well. 100µl of the HRP-conjugate reagent was added to each well, which was then sealed with an adhesive strip and left to incubate for 60 minutes at 37°C. Following aspiration and five rounds of washing, 400µl of wash solution was applied to each well. Each of the two chromogen solutions was applied in 50µl increments to each well. The micro-ELISA strip plate was placed out of direct sunlight, shaken gently, and incubated for 15 minutes at 37 °C. Fifty microliters of stop solution were added to each well. It became yellow after originally being blue. It changed the hue from blue to yellow. The optical density at 450 nm was measured using a microtiter plate reader in around 15 minutes. The quantity of unknown samples was ascertained using the standard curve. The standard curve was created by plotting the average optical density (450 nm) for each of the six standard concentrations.

Analytical Statistics

All statistical analyses were carried out using GraphPad Prism 7.0a. The data were expressed using mean ± SEM, which is the standard error of the mean. One-way analysis of variance was used to determine the statistical significance with p-values less than 0.05 (ANOVA) thought to be significant.

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III. RESULTS AND DISCUSSIONS

Lactate dehydrogenase (LDH) serves as an indirect diagnostic for angiogenesis, tumor, and hypoxia unfavorable prognosis in hepatocellular carcinoma [20] and plays a significant role in cellular energy production. The study indicated a significant rise in serum, and liver LDH levels in NDEA-induced rats, (Figures 1 and 2) with a significant decrease ($p < 0.001$) observed in the silymarin and sorafenib groups, indicating the role of silymarin in HCC treatment. Elevated LDH levels in the blood are typically used as indicators of tissue damage [21]. This study is in agreement with the research of [10] that discovered that crocin/sorafenib combination treatment significantly lowered serum LDH raised by Diethylnitrosamine

This study found that NDEA administration significantly increased serum MDA levels (Figure 3). However, treatment with 10 mg sorafenib and various concentrations of silymarin (50 mg silymarin and 75mg silymarin) significantly reduced MDA levels ($p < 0.001$). In the serum and liver (Figures 3 and 4), no significant differences were observed among the two silymarin treatment groups (Groups E and F). The elevated serum MDA levels indicate higher lipid peroxidation and oxidative damage in the liver. Lipid peroxidation triggered by NDEA contributes significantly to carcinogenesis and can produce toxic byproducts, like MDA, which targets biological components including DNA, resulting in genetic mutation and carcinogenicity [22]. The results reported are compatible with the studies by [23] and [8], who also reported increased liver MDA levels following NDEA administration.

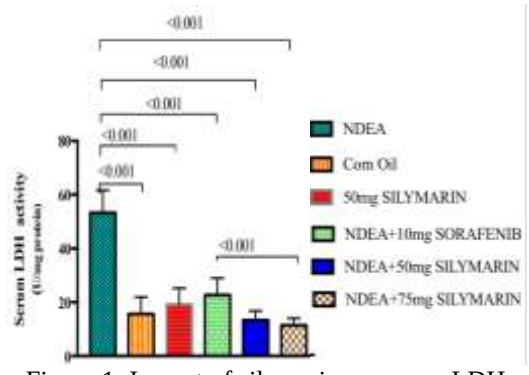

Figure 1: Impact of silymarin on serum LDH activity in the male Wistar rats with hepatocellular carcinoma caused by NDEA. Data are expressed in the form of means ± SD. #p < 0.05, ##p < 0.01, ###p < 0.001, values exhibit statistical significance.

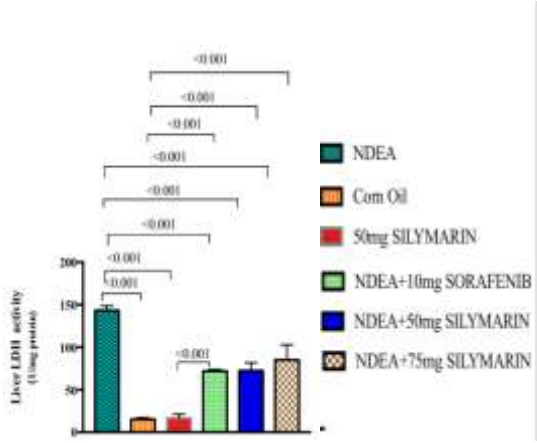

Figure 2: Impact of silymarin on liver LDH activity in the male Wistar rats with hepatocellular carcinoma caused by NDEA. Data are expressed in the form of means ± SD. #p < 0.05, ##p < 0.01, ###p < 0.001, values exhibit statistical significance.

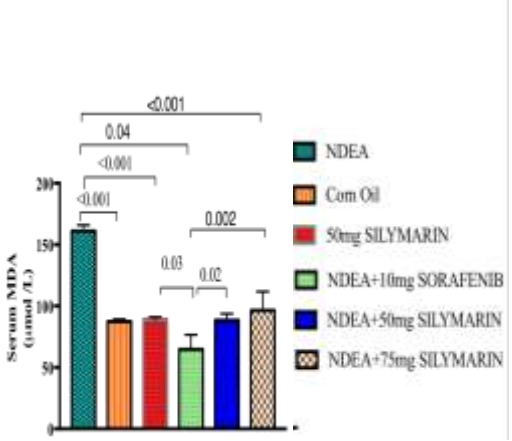

Figure 3: Impact of silymarin on serum MDA level in the male Wistar rats with hepatocellular carcinoma caused by NDEA. Data are expressed in the form of means ± SD. #p < 0.05, ##p < 0.01, ###p < 0.001, values exhibit statistical significance.

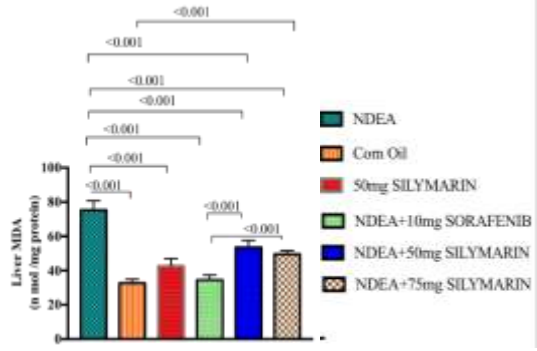

Figure 4: Impact of silymarin on liver MDA level in the male Wistar rats with hepatocellular carcinoma caused by NDEA. Data are expressed in the form of means ± SD. #p < 0.05, ##p < 0.01, ###p < 0.001, values exhibit statistical significance.

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Research has shown how NF-kB plays an essential regulator in the initial stages of neoplastic lesion development in the liver [24]. In this investigation, NDEA induced a considerable rise in NF-kB concentration in the serum of NDEA-induced, and liver as shown in Figures 5 and 6. However, treatments with silymarin, as well as sorafenib significantly ($p < 0.001$), reduced NF-kB protein levels induced by the NDEA. In the liver, this study revealed that in the liver there was no significant difference between the NDEA+50mg silymarin group and NDEA +10mg sorafenib group (Figure 6), whereas, treatment with 75mg silymarin attenuated NF-kB than 10mg sorafenib, a standard drug (Figure 5). This study agreed with [25] which explored the effects of silybum marianum whole extract in preventing renal carcinogenesis in Wistar rats. The researcher found out that silybum marianum whole extract downregulated NF-kB.

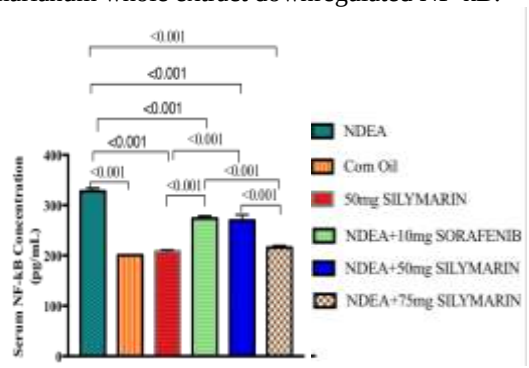

Figure 5: Impact of silymarin on serum NF-kB concentration in the male Wistar rats with hepatocellular carcinoma caused by NDEA. Data are expressed in the form of means ± SD. #p < 0.05, ##p < 0.01, ###p < 0.001, values exhibit statistical significance.

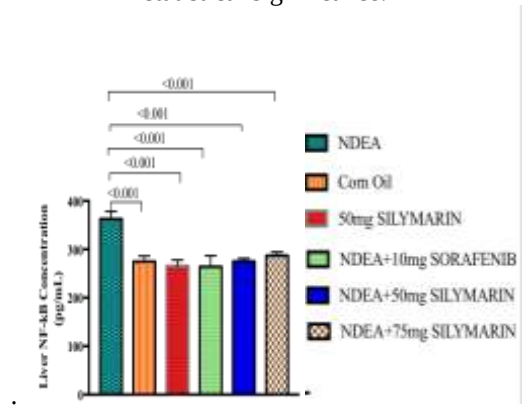

Figure 6: Impact of silymarin on liver NF-kB concentration in the male Wistar rats with hepatocellular carcinoma caused by NDEA. Data are expressed in the form of means ± SD. #p < 0.05, ##p < 0.01, ###p < 0.001, values exhibit statistical significance.

Serum cytokines play a critical function in mediating various pathological and physiological processes related to inflammation and cancer progression. NDEA elevated IL-6 in hepatocellular carcinoma, while administration of silymarin and sorafenib decreased the level of IL-6 (Figures 7 & 8). The concentration of serum IL-6 in NDEA-induced rats was up-regulated ($p< 0.001$) than all treated and negative control groups. Figure 7 shows that in serum, silymarin treatments were dose-dependent with 75mg of silymarin lowered IL-6 than sorafenib, a reference drug. However, in the liver, no significant difference was observed between different doses of silymarin and sorafenib (Figure 8). This result supports [8] finding, the study found out that NDEA up-regulation of IL-6 occurred in the liver, while spirulina and garlic downregulated IL-6 after treatment.

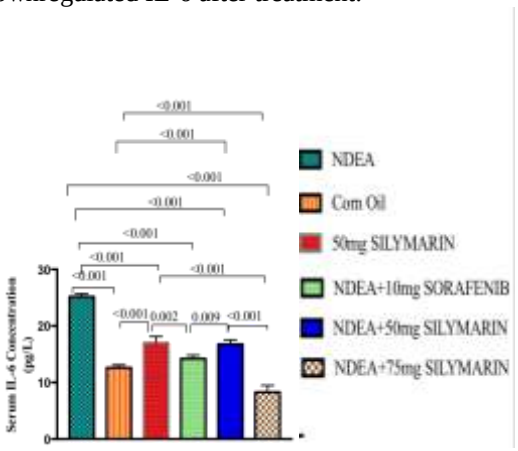

Figure 7: Impact of silymarin on serum IL-6 concentration in the male Wistar rats with hepatocellular carcinoma caused by NDEA Data are expressed in the form of means ± SD. #p < 0.05, ##p < 0.01, ###p < 0.001, values exhibit statistical significance.

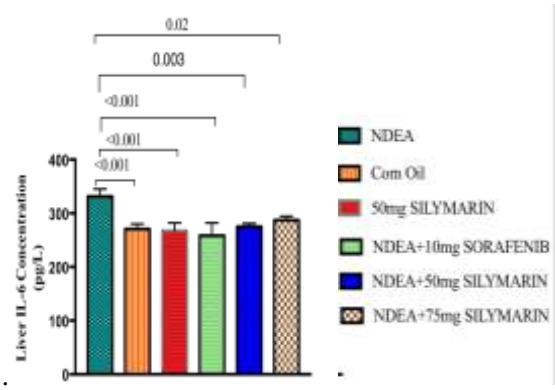

Figure 8: Impact of silymarin on liver IL-6 concentration in the male Wistar rats with hepatocellular carcinoma caused by NDEA. Data

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are expressed in the form of means ± SD. #p < 0.05, ##p < 0.01, ###p < 0.001, values exhibit statistical significance.

The NDEA-treated groups downregulated interleukin 10 (IL-10) as shown in Figures 9 & 10. All the treated groups up-regulated the IL-10 in serum and liver, (p< 0.001). No observable difference in all the treated groups. The increase in IL-10 can be seen as an effort to moderate hyperinflammation and protect against tissue damage. IL-10's primary role in infection is to suppress the organism's immunological response to pathogens and microbiota, therefore decreasing tissue damage and inflammation. It does this by suppressing the production of pro-inflammatory cytokines. This research is in support of [26]. [26] investigated the hepatoprotective properties of apigenin against liver damage both in vivo and in vitro. Their findings showed that apigenin upregulated IL-10.

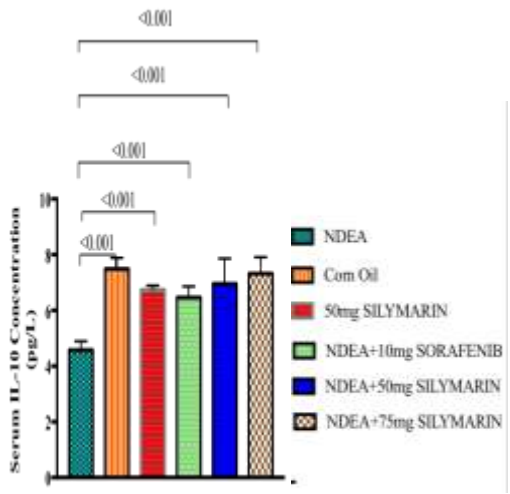

Figure 9: Impact of silymarin on serum IL-10 concentration in the male Wistar rats with hepatocellular carcinoma caused by NDE. Data are expressed in the form of means ± SD. #p < 0.05, ##p < 0.01, ###p < 0.001, values exhibit statistical significance.

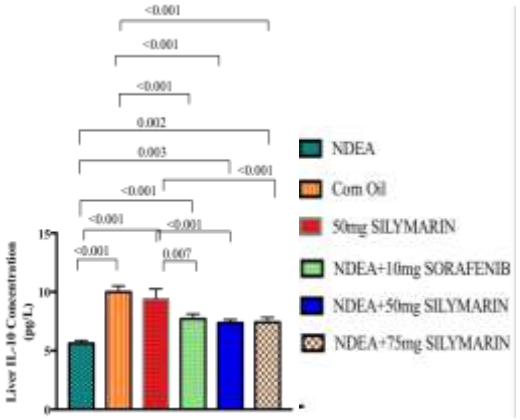

Figure 10: Impact of silymarin on liver IL-10 concentration in the male Wistar rats with hepatocellular carcinoma caused by NDEA. Data are expressed in the form of means ± SD. #p < 0.05, ##p < 0.01, ###p < 0.001, values exhibit statistical significance.

IV. CONCLUSION

This study offers significant insights regarding the function of silymarin in mitigating the progression of neoplastic lesions in the liver caused by the carcinogen NDEA. The findings demonstrate that NDEA significantly elevated the NF-kB expression, a key mediator engaged in early events promoting hepatocellular carcinogenesis. However, silymarin treatment effectively mitigated this elevation, indicating their potential as hepatoprotective agents against carcinogen-induced liver damage. Additionally, the study underscores the significance of cytokines like IL-10 and IL-6 in inflammation and cancer progression. NDEA led to the downregulation of IL-10, while silymarin treatment upregulated its expression. This indicates that silymarin may help regulate the immune response to reduce hyperinflammation and prevent tissue damage. Conversely, NDEA induced the upregulation of IL-6, which was subsequently reduced by silymarin and sorafenib treatments.
In conclusion, silymarin significantly lowered serum LDH, NF-kB, and IL-6 than sorafenib a reference drug.

V. APPRECIATION

I would like to express my heartfelt gratitude to ‘the Tertiary Education Trust Fund (TETFUND)’ for generously sponsoring this research work and the management of Kwara State College of Education, Oro. I am deeply appreciative of their belief in its potential impact. Thank you for enabling me to explore this vital area of study and for contributing to the

advancement of scientific knowledge that could benefit public health.

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