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Theme:

Single-Cell Nanoencapsulation for Immune Enhancement: An Experimental Study on the Immunomodulatory Effects of CuO Nanoparticles Bio-Conjugated with BCC in Cisplatin-Treated Rats

Presented by: BEN MOUSSA Sara and NESRATE Rim

Examining Committee:

President:	**Dr. AOUIMEUR Meriem**	**MCA**	**El-Oued University**
Examiner:	**Dr. YOUMBAI Asma**	**MCA**	**El-Oued University**
Supervisor:	**Pr. DEROUICHE Samir**	**Professor**	**El-Oued University**
Co-Supervisor:	**Dr. CHETEHOUNA Sara**	**M.A.B**	**El-Oued University**

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بسم الله الرحمن الرحيم
١٤٣٠

﴿ بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ ﴾

قال الله تعالى: ﴿ وَآخِرُ دَعْوَاهُمْ أَنِ الْحَمْدُ لِلَّهِ رَبِّ الْعَالَمِينَ ﴾ صدق الله العظيم — سورة يونس، الآية 10

الحمدُ للهِ حمدًا كثيرًا طيبًا مباركًا فيه، الحمدُ للهِ الذي بنعمته تتمّ الصالحات، وبفضله تتحقق الأمنيات، وما كان هذا النجاح ليتحقق لولا توفيقه وكرمه ولطفه. خمسة سنوات لم تكن سهلة، مليئة بالتعب والسهر والضغط واللحظات التي ظننت فيها أن الطريق أطول من قدرتي، لكن رحمة الله كانت دائمًا تسبق تعبي، وكان الأمل يعود إلى قلبي كلما أوشكت على الانطفاء. واليوم، وبعد رحلةٍ لم تكن قصيرة أبدًا، أقف بفخر وامتنان لأقول: لقد فعلتها... والحمد لله دائمًا وأبدًا.

أهدي هذا الإنجاز أولًا إلى نفسي، إلى تلك الروح التي قاومت بصمت، وصبرت رغم التعب، ولم تستسلم مهما اشتدت الأيام، إلى نفسي التي آمنت أن بعد كل تعب فرحًا يستحق الانتظار.

ثم إلى أغلى ما أملك في هذه الحياة، أمي الحبيبة سهام دردوري وأبي العزيز عبد الحق بن موسى، إلى من كانا دعائي المستجاب وسندي الحقيقي بعد الله، إلى من حملا عني ثقل الحياة بمحبةٍ لا تُوصف، وكانا النور في كل عتمة، والقوة في كل ضعف. كلمات الشكر لن تكفيكما أبدًا، فلكما مني كل الحب والامتنان، وأسأل الله أن يحفظكما ويديمكما تاجًا فوق رأسي.

إلى إخوتي وأخواتي: بلقاسم، أسيل، وروميصاء، أنتم دفء أيامي وفرح قلبي، ووجودكم كان دائمًا مصدر راحة وقوة لي.

وإلى جدي بلقاسم بن موسى وجدتي زهرة دقاشي رحمهما الله رحمةً واسعة، وأسكنهما فسيح جناته، وإلى جدتي فوزية دقاشي وجدي عمارة دردوري حفظهما الله وأطال في عمرهما، أهدي هذا النجاح عرفانًا لدعواتهم ومحبتهم التي رافقتني طوال الطريق.

إلى خالتي الغالية جيهان دردوري، وإلى جميع أفراد عائلتي من الأخوال دردوري والأعمام بن موسى، شكرًا لكل دعم، لكل دعوة صادقة، ولكل كلمة رفعت من عزيمتي يومًا و إلى قدوتي الدكتورة حسيبة شتحونة، التي كانت مثالًا للعِلم والاجتهاد والرقي، شكرًا لأنك كنتِ مصدر إلهام حقيقي لي، ولأن حضورك العلمي والإنساني زرع بداخلي الطموح والإصرار لأصبح أفضل في مستقبلي العلمي والمهني.

إلى أستاذي ومؤطري بروفيسور درويش سمير، ومساعدته استاذتي الدكتورة سارة شتحونة، جزيل الشكر والتقدير على كل التوجيهات والملاحظات القيمة، وعلى دعمكم العلمي والإنساني الذي كان له أثر كبير في إنجاز هذا العمل.

إلى جميع أساتذتي في الكلية، الذين علّموني، وآمنوا بقدراتي، وكان لكل واحد منهم أثرٌ جميل في تكويني العلمي والإنساني، مهما كتبت فلن أوفيكم حقكم، فلكم مني كل الاحترام والتقدير والعرفان.

وإلى مديري كريم حاوي، وكل الأساتذة والزملاء والموظفين في أكاديمية كريم، عائلتي الثانية التي احتضنتني بمحبة ودعم، شكرًا لكل لحظة تشجيع، ولكل موقف جميل ترك أثرًا طيبًا في قلبي، فوجودكم كان نعمة حقيقية في هذه الرحلة.

وإلى رفيقتي في هذا العمل، وثنائيتي الجميلة ريم نصرات، التي شاركتني تفاصيل هذه الرحلة بكل ما فيها من تعب وضغط وسهر وقلق، شكرًا لأنك كنتِ الرفيقة التي هوّنت عليّ الطريق، وشاركتني اللحظات الصعبة قبل الجميلة، فكان النجاح أجمل لأنك كنتِ جزءًا منه وإلى رفيقة الروح إيناس زهور فرجاني، وصديقتي الغالية نور الهدى بليلة و نجود قابوسة واكرام خادم الله وكل صديقاتي واخواتي ، شكرًا لأنكن كنتن دائمًا مصدر طاقة وطمأنينة ومحبة صادقة.

وفي الأخير، الحمد لله على هذا الإنجاز الذي جاء بعد تعبٍ طويل، والحمد لله على كل لحظة ضعف تحولت إلى قوة، وعلى كل دعوة حملتني نحو هذا اليوم. وأسأل الله أن يجعل هذا النجاح بدايةً لأيام أجمل، وأن يرزقني التوفيق فيما هو قادم.

سارة بن موسى

بسم الله الرحمان الرحيم

لم يكن الحلم يوما اختياري لكنّه أصبح أجمل أقداري

دخلتُ التخصّصَ وقلبي متردّدٌ، واليومَ أخرجُ منهُ وأنا به أفتخرُ

أحببتُ فيهِ تعبَ الليالي، وصرتُ أرى النجاحَ بعدَ المحالِ

الحمد لله عند البدء وعند الختام فما تناهى درب ولا ختم جيد ولا تم السعي الا بفضله فالحمدُ لله ها أنا اليومَ خريجةٌ بكلِّ فخرٍ ومحبة بعد خمس سنوات من التعب والسهر، وخلف كل لحظة ضعف كانت هناك قوة خفيّة تدفعني للاستمرار، أقف اليوم على عتبة النهاية، أحمل بين يدي ثمرة سنواتٍ كانت من أجمل وأصعب مراحل حياتي الدراسية ولاتمام هذا النجاح وبكل فخر وحب,اهدي نجاحي وتخرجي:

الى امي الحبيبة .... الروح التي كانت تدعو لي في كل صلاة، والقلب الذي احتواني حبا وصبرا وحنانا،والتي اوصاني الله بها خيرا والتي بها وصلت خريجة هذا اليوم

الى ابي الغالي, ذلك الرجل العظيم الذي كان سندي وقوتي وفخري،والذي احمل اسمه,وسندي بعد الله الذي علّمني أن الوصول يحتاج صبرا وإيمانا بالنفس،وحصد الاضواك في طريقي ليمهد لي درب العلم أطال الله عمرك وحفظك لي

الى روح جدتي الطاهرة.... رحمكِ الله رحمةً واسعة، كنتِ سندي ودعواتكِ كانت ترافقني دائماً, وكان حلمكِ أن تشهدي يوم تخرّجي وها أنا اليوم أُتمّ الحلم، لكن القلب يفتقدكِ كثيراً

إلى إخوتي الأحبّة... أختي الكبرى هناء كنتِ لي الأخت والصديقة والسند في كل المراحل, وإلى أخي علي شكراً لوجودك ودعمك الدائم وفقك الله في الباكالوريا واخوتي الى اخر العنقود احمد نذير حفظكم الله

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Abstract

This study aimed to biosynthesize a Nano-therapeutic vaccine to alleviate cisplatin-induced immunosuppression and toxicity in rats. Copper oxide nanoparticles (CuONPs) were synthesized using human serum albumin (HSA) as a stabilizing agent and bio-conjugated with breast cancer cell lysates (BCC) to generate CuONPs-BCC, a nanomaterial designed as a therapeutic vaccine candidate. The prepared copper nanoparticles were characterized using UV–Visible spectroscopy, Fourier Transform Infrared spectroscopy (FTIR), X-Ray Diffraction (XRD), Scanning Electron Microscopy (SEM), and Energy Dispersive X-ray (EDX) analysis. Further, *in-vitro* biological activities of prepared nanoparticles were assessed, antioxidant activity was assessed using the ferric reducing antioxidant power (FRAP) assay, while anti-inflammatory potential was investigated through anti-hemolytic and protein denaturation inhibition assays. For *in-vivo* study, female Wistar albino rats were randomly divided into four groups (n = 5): Control rat group, cisplatin-treated rat group, immune-depressed rat group treated with CuONPs-BCC, and immune-depressed rat group treated with BCC. The rats were received treatments via intraperitoneal route for three weeks. Cisplatin was used to induce immunodepression and organ toxicity. Different investigations were performed, including hematological parameters; biochemical parameters, such as blood glucose and C-reactive protein (CRP); oxidative stress biomarkers in different organs, including lipid peroxidation and antioxidant defense markers; and histopathological examination of liver, kidney, heart, and spleen tissues. Immunological evaluation was also carried out to assess the immunomodulatory effects of the therapeutic formulation. The obtained results confirmed the successful formation of stable copper nanoparticles with very small crystallite sizes, approximately 8 nm for CuONPs and 8.3 nm for CuONPs-BCC .For *in-vitro* study results, in the FRAP assay, CuONPs-BCC exhibited the strongest antioxidant activity with an IC_{50} value of 66 µg/mL, followed by CuONPs (80 µg/mL. Anti-hemolytic activity results showed that CuONPs displayed the highest protective effect against hemolysis with an IC_{50} value of approximately 7 µg/mL, outperforming CuONPs-BCC (14 µg/mL). In contrast, the protein denaturation assay revealed that CuONPs-BCC possessed the strongest inhibitory activity, with an IC_{50} value (70 µg/mL). On the other hand, toxicity test results showed that the nanoparticles can be used safely, as they did not exhibit any toxicity to Rats at high concentrations. In *vivo* findings demonstrated that cisplatin administration caused significant increases ($p < 0.05$) in blood glucose and C-reactive protein (CRP) levels, associated with alterations in hematological parameters. Furthermore, an immunosuppressive effect was observed, as evidenced by changes in leukocyte counts and IgG levels. Oxidative stress markers were also significantly affected ($p < 0.05$) by elevated malondialdehyde (MDA) levels, along with decreased reduced glutathione (GSH) content and superoxide dismutase (SOD) activity in study organs. Histopathological analysis also showed severe tissue damage characterized by necrosis, inflammatory infiltration, hemorrhage, and disruption of normal tissue architecture in the liver, kidney, heart, and spleen. In contrast, treatment with CuNPs-BCC markedly enhanced immune function, improved hematological and biochemical

parameters, and restored antioxidant balance compared with the cisplatin-treated group. A significant improvement in tissue architecture, along with a reduction in inflammatory and degenerative lesions, was observed in all examined organs. Although the BCC-treated group also exhibited signs of recovery, its protective effects were less pronounced than those achieved with CuONPs-BCC treatment. In conclusion, the present findings suggest that CuONPs-BCC possesses promising antioxidant, anti-inflammatory, immunomodulatory, and cytoprotective activities, making it a potential therapeutic vaccine candidate for alleviating immunosuppression during cancer chemotherapy.

Keywords: CuONP-BCC, Cisplatin, Immunosuppression, Therapeutic vaccine, Antioxidant, Anti-inflammatory, Rats.

ملخص

هدفت هذه الدراسة إلى تصنيع لقاح نانوي علاجي حيويًا للتخفيف من تثبيط المناعة والسمية الناجمين عن السيسبلاتين في الفئران. تم تصنيع جسيمات أكسيد النحاس النانوية (CuO NPs) باستخدام ألبومين مصل الدم البشري (HSA) كعامل استقرار، وتم ربطها حيويًا بمستخلصات خلايا سرطان الثدي (BCC) لإنتاجCuO NPs-BCC ، وهي مادة نانوية مصممة كمرشح للقاح علاجي. تم توصيف جسيمات النحاس النانوية المُحضّرة باستخدام مطيافية الأشعة فوق البنفسجية والمرئية، ومطيافية الأشعة تحت الحمراء بتحويل فورييه(FTIR) ، وحيود الأشعة السينية(XRD) ، والمجهر الإلكتروني الماسح(SEM) ، وتحليل تشتت طاقة الأشعة السينية .(EDX)علاوة على ذلك، تم تقييم الأنشطة البيولوجية للجسيمات النانوية المُحضّرة في المختبر، وقُيِّم النشاط المضاد للأكسدة باستخدام اختبار قدرة اختزال الحديد المضاد للأكسدة(FRAP) ، بينما تم التحقق من القدرة المضادة للالتهابات من خلال اختبارات تثبيط انحلال الدم وتثبيط تمسخ البروتين. وللدراسة في الجسم الحي، قُسِّمت إناث فئران ويستار البيضاء عشوائيًا إلى أربع مجموعات (ن = 5): مجموعة فئران ضابطة، ومجموعة فئران مُعالَجة بالسيسبلاتين، ومجموعة فئران مُثبَّطة المناعة مُعالَجة بـCuONPs-BCC ، ومجموعة فئران مُثبَّطة المناعة مُعالَجة بـ BCC. تلقت الفئران العلاجات عن طريق الحقن داخل الصفاق لمدة ثلاثة أسابيع. استُخدم السيسبلاتين لتحفيز تثبيط المناعة وسمية الأعضاء. أُجريت فحوصات مختلفة، شملت معايير الدم؛ ومعايير كيميائية حيوية، مثل جلوكوز الدم وبروتين سي التفاعلي(CRP) ؛ ومؤشرات الإجهاد التأكسدي في أعضاء مختلفة، بما في ذلك بيروكسيد الدهون ومؤشرات الدفاع المضاد للأكسدة؛ وأُجري فحص نسيجي مرضي لأنسجة الكبد والكلى والقلب والطحال. كما أُجري تقييم مناعي لتقييم التأثيرات المناعية للصيغة العلاجية. وأكدت النتائج المُتحصل عليها نجاح تكوين جزيئات نانوية نحاسية مستقرة ذات أحجام بلورية صغيرة جدًا، تبلغ حوالي 8 نانومتر لجزيئات أكسيد النحاس النانوية (CuONPs) و8.3 نانومتر لجزيئات أكسيد النحاس النانوية المُركبة على بنية الخلايا القاعدية .(CuONPs-BCC)أظهرت نتائج الدراسة المخبرية، في اختبارFRAP ، أن جسيمات أكسيد النحاس النانوية المرتبطة بـ BCC (CuONPs-BCC)تتمتع بأقوى نشاط مضاد للأكسدة بقيمة IC_{50} تبلغ 66 ميكروغرام/مل، تليها جسيمات أكسيد النحاس النانوية (CuONPs)بتركيز 80 ميكروغرام/مل. وأظهرت نتائج النشاط المضاد لانحلال الدم أن جسيمات أكسيد النحاس النانوية (CuONPs) تتمتع بأعلى تأثير وقائي ضد انحلال الدم بقيمة IC_{50} تبلغ حوالي 7 ميكروغرام/مل، متفوقةً بذلك على جسيمات أكسيد النحاس النانوية المرتبطة بـ (CuONPs-BCC) BCC بتركيز 14 ميكروغرام/مل. في المقابل، كشف اختبار تمسخ البروتين أن جسيمات أكسيد النحاس النانوية المرتبطة بـ (CuONPs-BCC) BCC تمتلك أقوى نشاط تثبيطي، بقيمة IC_{50} تبلغ 70 ميكروغرام/مل. من ناحية أخرى، أظهرت نتائج اختبار السمية أن الجسيمات النانوية آمنة للاستخدام، حيث لم تُظهر أي سمية للفئران عند التركيزات العالية. وأظهرت النتائج في الجسم الحي أن إعطاء السيسبلاتين تسبب في زيادات كبيرة ($p < 0.05$) في ارتبطت مستويات سكر الدم وبروتين سي التفاعلي (CRP) بتغيرات في المؤشرات الدموية. علاوة على ذلك، لوحظ تأثير مثبط للمناعة، كما يتضح من التغيرات في تعداد الكريات البيضاء ومستويات الغلوبولين المناعي .IgG كما تأثرت مؤشرات الإجهاد التأكسدي بشكل ملحوظ ($p < 0.05$) بارتفاع مستويات مالونديالدهيد(MDA) ، إلى جانب انخفاض محتوى الجلوتاثيون المختزل (GSH) ونشاط إنزيم ديسموتاز الفائق (SOD) في الأعضاء المدروسة. أظهر التحليل النسيجي المرضي أيضًا تلفًا شديدًا في الأنسجة يتميز بالنخر، والتسلل الالتهابي، والنزيف، واضطراب بنية الأنسجة الطبيعية في الكبد والكلى والقلب والطحال. في المقابل، عزز العلاج بجزيئات النحاس النانوية المرتبطة بـ BCCبشكل ملحوظ وظيفة المناعة، وحسّن المؤشرات الدموية والكيميائية الحيوية، وأعاد توازن مضادات الأكسدة مقارنةً بالمجموعة المعالجة بالسيسبلاتين. لوحظ تحسن كبير في بنية الأنسجة، إلى جانب انخفاض في الآفات الالتهابية والتنكسية، في جميع الأعضاء التي تم فحصها. على الرغم من أن المجموعة المعالجة بـ BCC أيضًا أظهرت علامات التعافي، إلا أن تأثيراته الوقائية كانت أقل وضوحًا من تلك التي تم الحصول عليها باستخدام علاج .CuONPs-BCC في الختام، تشير النتائج الحالية إلى أن CuONPs-BCC يمتلك خصائص واعدة كمضاد للأكسدة، ومضاد للالتهابات، ومعدل للمناعة، وواقي للخلايا، مما يجعله مرشحًا محتملاً للقاح علاجي لتخفيف تثبيط المناعة أثناء العلاج الكيميائي للسرطان.

الكلمات المفتاحية: CuONP-BCC، سيسبلاتين، تثبيط المناعة، لقاح علاجي، مضاد للأكسدة، مضاد للالتهابات، فئران.

ABBREVIATIONS LIST

Abbreviation	Full Term
BCC	Breast Cancer Cell Lysate
CuONPs	Copper Oxide Nanoparticles
CuONPs-BCC	Copper Oxide Nanoparticles Bio-Conjugated with Breast Cancer Cell
HSA	Human Serum Albumin
FTIR	Fourier Transform Infrared Spectroscopy
XRD	X-Ray Diffraction
SEM	Scanning Electron Microscopy
EDX	Energy Dispersive X-ray Analysis
UV-Vis	Ultraviolet–Visible Spectroscopy
FRAP	Ferric Reducing Antioxidant Power
CRP	C-Reactive Protein
IgG	Immunoglobulin G
MDA	Malondialdehyde
GSH	Reduced Glutathione
SOD	Superoxide Dismutase
PBS	Phosphate Buffered Saline
TCA	Trichloroacetic Acid
EDTA	Ethylenediaminetetraacetic Acid
BSA	Bovine Serum Albumin
DTNB	5,5′-Dithiobis-(2-Nitrobenzoic Acid)
NBT	Nitro Blue Tetrazolium
TBA	Thiobarbituric Acid
BHT	Butylated Hydroxytoluene
NaOH	Sodium Hydroxide
$CuSO_4 \cdot 5H_2O$	Copper Sulfate Pentahydrate
ROS	Reactive Oxygen Species
NK Cells	Natural Killer Cells
APCs	Antigen-Presenting Cells
PRRs	Pattern Recognition Receptors
TLRs	Toll-Like Receptors
MHC-II	Major Histocompatibility Complex Class II

DCIS	Ductal Carcinoma In Situ
LCIS	Lobular Carcinoma In Situ
BRCA1	Breast Cancer Gene 1
BRCA2	Breast Cancer Gene 2
AKI	Acute Kidney Injury
NF-κB	Nuclear Factor Kappa B
TNF-α	Tumor Necrosis Factor Alpha
IL-6	Interleukin-6
AMPARs	α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid Receptors
NMDARs	N-Methyl-D-Aspartate Receptors
HCl	Hydrochloric Acid
K_2HPO_4	Dipotassium Hydrogen Phosphate
KH_2PO_4	Potassium Dihydrogen Phosphate

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Introduction

Introduction

Breast cancer is one of the most frequently diagnosed cancers and remains a leading cause of cancer-related mortality among women worldwide (Siegel et al., 2024). Despite the considerable advances achieved in early diagnosis and therapeutic strategies, breast cancer continues to represent a major global health challenge due to tumor heterogeneity, metastasis, and treatment resistance (Liu et al., 2023). Conventional therapeutic approaches, including surgery, radiotherapy, chemotherapy, and hormonal therapy, are widely used in breast cancer management; however, these treatments are often associated with serious adverse effects and limited selectivity toward cancer cells (Zhou et al., 2024). Chemotherapeutic drugs, in particular, can damage healthy tissues and induce systemic toxicity because they are unable to specifically target tumor cells (Srinivasan et al., 2022). In addition, prolonged chemotherapy may lead to immunosuppression, oxidative stress, and multidrug resistance, which significantly reduce treatment efficiency and negatively affect patient quality of life (Liu et al., 2023).

Cancer vaccine therapy has emerged as a promising immunotherapeutic strategy for breast cancer by stimulating the immune system to recognize and eliminate tumor cells. Through enhanced antigen presentation and activation of cytotoxic T lymphocytes, cancer vaccines can induce durable antitumor immune responses and long-term immunological memory (Morisaki et al., 2024; Mantooth et al., 2024). Tumor immune evasion and the immunosuppressive tumor microenvironment remain major challenges, prompting ongoing efforts to improve vaccine delivery systems and combination immunotherapies (Binnewies et al., 2018; Sharma et al., 2021).

In recent years, nanotechnology has emerged as one of the most promising approaches in cancer therapy due to its ability to improve drug delivery and therapeutic targeting (Zhou et al., 2024). Nanoparticles possess unique physicochemical properties, including nanoscale size, large surface-area-to-volume ratio, tunable surface chemistry, and enhanced permeability and retention (EPR) effect, which allow preferential accumulation within tumor tissues (Wang et al., 2023). These characteristics improve drug stability, increase bioavailability, prolong circulation time, and reduce systemic toxicity compared with conventional therapeutic formulations (Zhou et al., 2024).

Among the different nanomaterials investigated in cancer treatment, copper nanoparticles have attracted considerable attention because of their multifunctional biological activities. Copper nanoparticles exhibit antioxidant, antimicrobial, anti-inflammatory, and anticancer properties through their redox activity and interactions with biological molecules (Vodyashkin et al., 2024). Several studies reported that copper nanoparticles can selectively induce oxidative stress in tumor cells, leading to mitochondrial dysfunction, DNA damage, and apoptosis (Yaqoob et al., 2023). Furthermore, they can protect immune

cells from oxidative stress and improve their survival within the hostile tumor environment, offering promising opportunities for safer and more effective anticancer therapies (Sharma et al., 2021; Mitragotri et al., 2022).

Among advanced nanotechnology-based strategies, the use of snail-derived biomaterials in nanotechnology has recently gained considerable attention in biomedical research (Gomes et al., 2023). Snail mucus contains a rich composition of bioactive molecules such as glycoproteins, polysaccharides, and antioxidants, which can be engineered at the nanoscale to form biocompatible and bioadhesive nanostructures for therapeutic applications (Silva et al., 2024). These snail-based nanomaterials exhibit strong protective and regenerative properties, making them suitable for enhancing cell stability, wound healing, and controlled drug delivery systems (Ferreira et al., 2023). In addition, snail mucus–derived nanostructures have been reported to reduce oxidative stress and improve cellular protection against environmental and biochemical damage while maintaining normal cellular functions (Gomes et al., 2023). Recent studies also suggest that integrating snail-based biomaterials with nanotechnology platforms can enhance immune modulation and improve therapeutic efficiency in cancer-related applications by supporting targeted delivery and reducing systemic toxicity (Ferreira et al., 2023).

Therefore, the present study focuses on the application of single-cell nanocapsulation using engineered nanoparticle systems as a novel strategy for enhancing the immune system against cisplatin chemotherapy drug. This work aims to investigate the biological potential of functionalized nanostructures in improving immune responses, reducing cellular damage, and enhancing anticancer activity compared with conventional therapeutic approaches.

Taking into consideration all the previous outcomes, we found that the most appropriate approach for maintaining a comprehensive study was to conduct the following two parts:

In vitro part: This part consisted of the synthesis of copper nanoparticles using HSA, coupled with breast cancer snail cells, followed by their characterization and evaluation of their biological properties.

In vivo part: This part involved the evaluation of the nanotherapeutic efficacy of biosynthesized CuNPs coupled with breast cancer snail cells (CuONPs-BCC) as a novel therapeutic system against chemotherapy-induced immunosuppression, as well as the physiological and histological alterations induced by experimental immunosuppression using cisplatin in rats.

First part:

Bibliographic Synthesis

Chapter I:

Breast Cancer

Chapter I: Breast Cancer

1. Epidemiology of Breast Cancer

Breast cancer is one of the most common malignancies among women and represents a major public health concern. In France, approximately 50,000 new cases were reported in 2008, making breast cancer the second most common cancer overall and accounting for nearly one-third of all newly diagnosed cancers in women. The standardized incidence rate was estimated at 100 cases per 100,000 population, with a median age at diagnosis of 61 years, while cases occurring before the age of 40 remain relatively rare. Over the past 25 years, the incidence of breast cancer has increased steadily and significantly. Despite this rise in incidence, mortality rates have started to decline since 2000, mainly due to improvements in treatment strategies and earlier detection through expanded screening programs. Breast cancer remains the leading cause of cancer-related deaths among women, representing 18.9% of cancer mortality, with a standardized mortality rate of 17.7 per 100,000 and 11,201 deaths reported in France in 2005. Moreover, the overall 5-year survival rate is approximately 80%, while the 10-year survival rate reaches nearly 70% across all stages combined (Loriot Y & Mordant P., 2011).

2. General Information about Cancer

2.1. Definition of Cancer

Cancer is an abnormal growth of cells caused by multiple changes in gene expression leading to dysregulated balance of cell proliferation and cell death and ultimately evolving into a population of cells that can invade tissues and metastasize to distant sites, causing significant morbidity and, if untreated, death of the host. (Ruddon, R. W., 2007).

2.2. Carcinogenesis

The carcinogenic process is counteracted by effective defence mechanisms in the cell, tissue and the organism. With regard to tissue, the mechanisms that govern embryogenesis and direct tissue repair after injury appear to play also an important role in the control of cell proliferation (Beachy et al., 2004). This is particularly important when a transformed cell is surrounded by normal cells that appear to be able to inhibit its proliferation. Tissue disorganization by inflammation or by the death of a large proportion of cells is often associated with the escape of the initiated cells and the emergence of a clone of pre-neoplastic–neoplastic cells. The effectiveness of immunosurveillance is also shown by the large

increase in the incidence of several types of cancers among immunodepressed people. (Tubiana, M., 2008).

2.3. The hallmarks of cancer

The hallmarks of cancer comprise six biological capabilities acquired during the multistep development of human tumors. The hallmarks constitute an organizing principle for rationalizing the complexities of neoplastic disease. They include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. Underlying these hallmarks are genome instability, which generates the genetic diversity that expedites their acquisition, and inflammation, which fosters multiple hallmark functions (Negrini et al., 2010). Conceptual progress in the last decade has added two emerging hallmarks of potential generality to this list—reprogramming of energy metabolism and evading immune destruction. In addition to cancer cells, tumors exhibit another dimension of complexity: they contain a repertoire of recruited, ostensibly normal cells that contribute to the acquisition of hallmark traits by creating the ‘‘tumor microenvironment’’ (Quail & Joyce, 2013). Recognition of the widespread applicability of these concepts will increasingly affect the development of new means to treat human cancer. (Hanahan, D., & Weinberg, R. A., 2011).

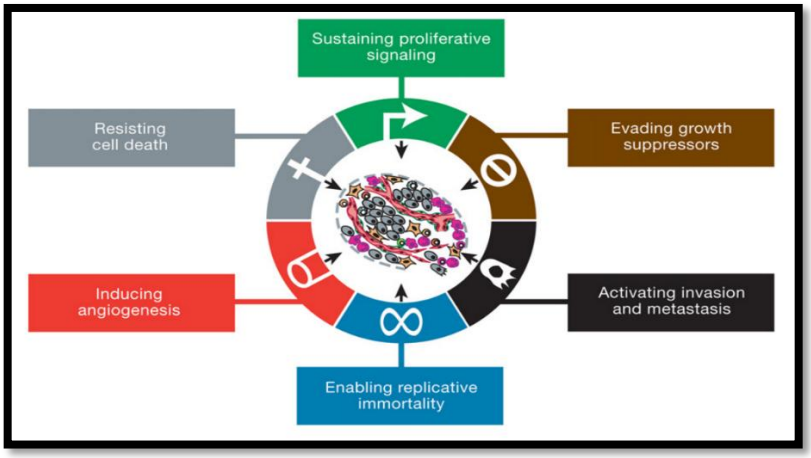


Figure 1: The Hallmarks of Cancer This illustration encompasses the six hallmark capabilities. (Hanahan, D., & Weinberg, R. A., 2011)

2.4. Breast

Breast is accessory gland of the skin. They exist in both sexes but remain rudimentary in males throughout life. The breasts are mainly present in the superficial fascia of the anterior thoracic wall except for the axillary tail of Spence which passes through the deep fascia to reach the apex of the axilla

(Drake et al., 2019). Between the breast and the deep fascia is present a loose connective tissue plane known as the submammary or retromammary space. Each breast consists of 15–20 lobes, that are drained by a lactiferous duct (Gupta, M., & Goyal, N.,2022).

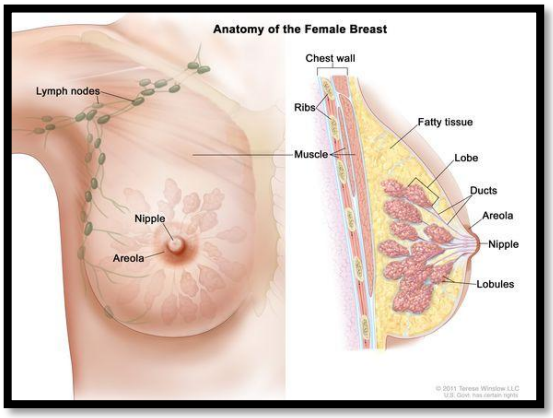


Figure 2: Anatomy of the Female Breast(Gupta, M., & Goyal, N.,2022).

2.5. Breast Cancer

Breast cancer is a malignant tumor originating from the breast ductal and lobules epithelium. Clinicopathological factors in breast cancer such as age, sex, location, laterality, histopathological type, grade, and lymph vascular invasion are known affect the prognosis. (Naqia, M., Darwin, E., & Tofrizal, T., 2025), it is characterized by the development of a tumor originating from glandular cells, with different names assigned based on the specific cells affected. The tumor's growth is uncontrolled by the body, expanding chaotically throughout the gland. (Sam, S et *al*., 2024).

3. Types of Breast Cancer

3.1. In Situ Carcinoma

Malignant epithelial proliferation occurs within the lumen of the lactiferous ducts, referred to as **ductal carcinoma in situ (DCIS)** (accounting for 85–90% of in situ carcinomas), or within the acini located in the lobules, referred to as **lobular carcinoma in situ (LCIS)** (10–15%). However, the basement membrane remains intact, and there is no invasion. (Loriot & Mordant, 2011).

3.2. Invasive (Infiltrating) Carcinoma

This is a cancer that invades breast tissue by breaching the basement membrane. Several histological types exist:

a. Invasive ductal adenocarcinoma (75%)

This is the most common type. Tumor cells are arranged in cords, solid clusters, and glandular formations. (Loriot & Mordant, 2011).

b. Invasive lobular adenocarcinoma (5–10%)

Carcinoma cells are arranged in a single-file pattern, often showing a target-like appearance around the lactiferous ducts. The nuclei are uniform, and the mitotic count is low. Immunohistochemically, there is a loss of E-cadherin expression. (Loriot & Mordant, 2011).

3.3. Tubular Adenocarcinoma

Carcinoma cells are arranged exclusively in glandular structures. The nuclei are regular, and the mitotic rate is low. This type of cancer is associated with a favorable prognosis. (Loriot & Mordant, 2011).

4. Risk Factors

- Personal or family history of breast cancer increases the relative risk when there is a first-degree family history (mother, sister, or daughter) **(Patel, 2024).**
- Approximately 5% of breast cancers are associated with mutations in the BRCA1 and BRCA2 genes **(Narod & Foulkes, 2004).**
- Bilateral breast cancer, early-onset breast cancer, family history of breast or ovarian cancer, male breast cancer, and medullary histological type are suggestive of hereditary predisposition **(Narod & Foulkes, 2004).**
- Breast cancer is an estrogen-dependent cancer **(Yager & Davidson, 2006).**
- Endogenous factors include early puberty, nulliparity, late first pregnancy, absence of breastfeeding, and late menopause **(Mao et al., 2023).**
- Exogenous factors include hormone replacement therapy and oral contraceptive use **(Yiallourou et al., 2024).**
- Atypical ductal or lobular hyperplasia is associated with an increased risk of breast cancer **(Kader et al., 2018).**
- Hyperplasia without atypia moderately increases breast cancer risk **(Dupont & Page, 1985).**
- Exposure to ionizing radiation is a recognized risk factor for breast cancer **(Ronckers et al., 2005).**

- High socioeconomic status has been associated with increased breast cancer incidence **(Danø et al., 2003).**

5. Cancer Treatment

Cancer treatment includes different methods such as surgery, chemotherapy, radiotherapy, and immunotherapy to remove or control cancer. (Peters, M.,2024)**.**

chemotherapy is not always effective in various types of cancer, a significant improvement in progression-free and overall survival has been observed. Polychemotherapy regimens are used in various types of malignancies, as they act against malignant cells at different phases of their cell cycle. (Chrysanthakopoulos, N. A., & Vryzaki, E.,2022).

Surgery remains the primary curative option for many early stage cancers. However, it is increasingly being integrated with other modalities in both neoadjuvant (pre-surgical) and adjuvant (post-surgical) settings. Neoadjuvant therapies can shrink tumors, making them more amenable to surgical resection, while adjuvant therapies can help eliminate any residual disease. Multimodal approaches involving surgery have demonstrated particular success in treating cancers of the breast. (Peters, M.,2024).

Radiotherapy is delivered for purposes of local control, but can also exert systemic effect on remote and non-irradiated tumor deposits, which is called abscopal effect. The view of Radiotherapy as a simple local treatment has dramatically changed in recent years, and it is now widely accepted that Radiotherapy can provoke a systemic immune response. (Zhang, Z., Liu, X., Chen, D., & Yu, J., 2022).

Immunotherapy has been widely used in cancer treatment in recent years and functions by stimulating the immune system to kill tumor cells. (Li, X.,et al.,2025).

Therapeutic cancer vaccines have undergone a resurgence in the past decade. A better understanding of the breadth of tumour-associated antigens, the native immune response and development of novel technologies for antigen delivery has facilitated improved vaccine design. (Saxena, M., Van Der Burg, S., Melief, C., & Bhardwaj, N.,2021) . it aims to expand and activate antigen-specific T cells for the targeted elimination of cancer cells. While early clinical trials faced challenges due to suboptimal antigen-specific T-cell activation, recent advancements in antigen discovery and vaccine platform engineering have revitalized the field. This review provides a comprehensive overview of key tumor antigens, including tumor-associated antigens, viral oncoprotein antigens, neoantigens, and cryptic antigens, with a focus on their immunogenicity and therapeutic potential.(Peng, K., Zhao, X., Fu, Y., & Liang, Y., 2025).

Chapter II:

Immunity and Cancer Vaccination

Chapter II: Immunity and Cancer Vaccination

1. Overview of the Immune System in Cancer

The immune response system contributes to the body's defense against infection, toxic or allergenic substances and is concerned with the recognition of tumor cells. In responding to a challenge, the immune system is able to distinguish the body's own cells and components (self) via the major histocompatibility complex (HLA-DR) class 1 from cells that are foreign (non-self). The abnormalities of the immune response is demonstrated in the immunodeficiency diseases (congenital and acquired), the hypersensitivity reactions that may be involved in producing autoimmune diseases and the switching-off of T cell function by cancer cells. (Weledji, E. P.,2025).

2. Tumor immunology

Tumor immunology is a critical area of cancer research that focuses on the interplay between the immune system and tumor cells. The immune system has the ability to recognize and eliminate cancer cells through various immune cells, such as cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. However, tumors have developed sophisticated mechanisms to evade immune detection, such as the loss of tumor antigen expression, recruitment of immunosuppressive cells, and upregulation of immune checkpoints. These immune evasion strategies allow tumors to grow uncontrollably and metastasize. (Kurtz, D. M., & Gambhir, S. S.,2014).

3. Cancer vaccines definition

Vaccination seeks to stimulate a protective immune response to a specific infectious pathogen by generating antibodies and activating certain cellular components (Canouï & Launay, 2019). This method is used in healthy people to impart protection against illness (Seneff et *al*., 2022). Vaccinations can be used to prevent disease (prophylactic vaccinations) or treat it (therapeutic vaccines) (Delany et *al*., 2014), making them one of the most effective methods for infectious disease prevention (Rambe et *al*., 2015).

4. Vaccine types

Cancer vaccines crucial in the immunotherapeutic landscape, are bifurcated into preventive and therapeutic types, both integral to combating oncogenesis. Preventive cancer vaccines, like those against HPV and HBV, reduce the incidence of virus-associated cancers, while therapeutic cancer vaccines aim

to activate dendritic cells and cytotoxic T lymphocytes for durable anti-tumor immunity. (Lei, W., Zhou, K., Lei, Y., Li, Q., & Zhu, H. ,2025).

Preventive (prophylactic) vaccines are instrumental in safeguarding healthy individuals against cancers linked to certain viruses, analogous to how the influenza vaccine protects against viral infection. The efficacy of these vaccines is contingent upon their administration prior to viral exposure. These cancer vaccines exemplify the importance of preventive measures in cancer control strategies, highlighting a shift towards interventions that can prevent rather than treat the disease, thus potentially saving millions of lives globally .(Lei, W., Zhou, K., Lei, Y., Li, Q., & Zhu, H. ,2025).

therapeutic vaccines aim to activate the immune system to recognize and eradicate cancer cells. These malignant cells possess distinct proteins, or antigens, which distinguish them from normal cells. Therapeutic cancer vaccines enhance the immune reaction by utilizing either non-specific pro-inflammatory agents and adjuvants to amplify existing anti-tumor defenses, or by triggering a new immune response targeted at specific tumor-associated antigens .(Lei, W., Zhou, K., Lei, Y., Li, Q., & Zhu, H. ,2025).

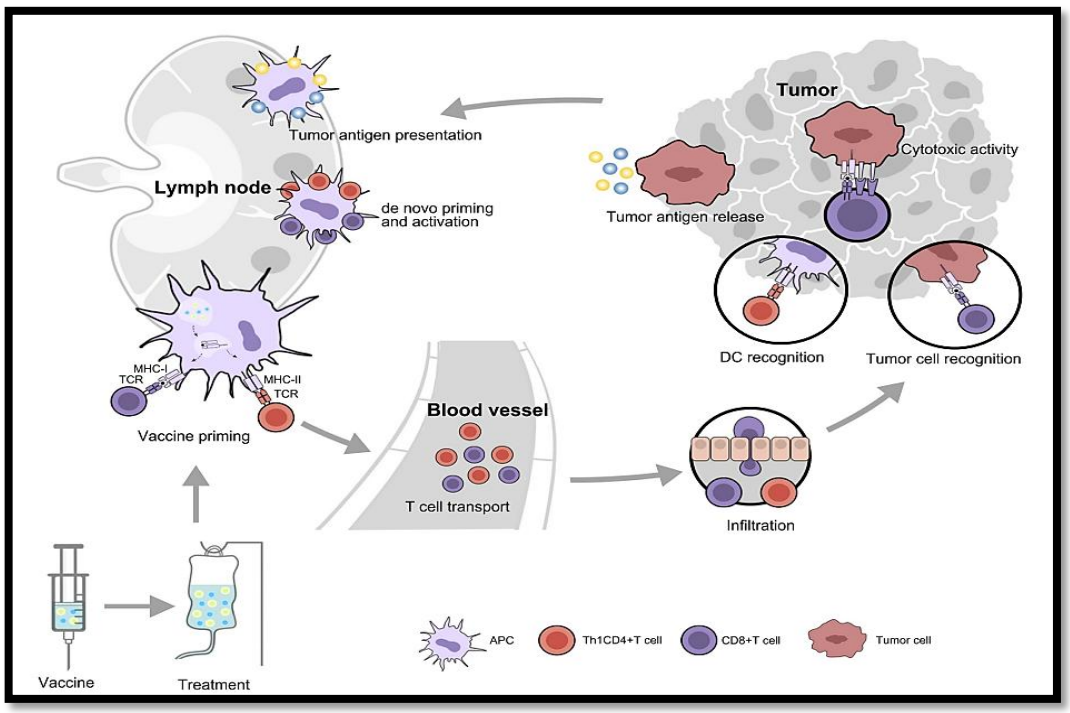


Figure 3: Immune response induced by therapeutic cancer vaccines. (Lei, W., Zhou, K., Lei, Y., Li, Q., & Zhu, H. ,2025).

5. Mechanism of action of a vaccine

Vaccines are intended to imitate infections and stimulate individual immunity to illness (Yadav et al., 2018). The innate immune system is the primary defense against foreign chemicals, reacting in hours with little specificity or memory (Vetter et al., 2018). Vaccination is intended to protect against infectious illnesses by stimulating immune responses to nonpathogenic microbial forms or components. The vaccine's active component, an immunogen, induces a protective response that includes both innate and adaptive immunity (Miot et al., 2019).There are two sorts of reactions: primary responses, which occur immediately after the first injection, and secondary responses, which are triggered by the second injection, or booster, which happens more than a month later. The major response is a complicated interaction of innate and adaptive immunity (Autran et al., 2016; Zepp, 2016; Moser & Leo, 2010).

Antigen-presenting cells (APCs) deliver the antigenic preparation to CD4+ T cells, initiating the vaccination response. The vaccine formulation affects this first phase as well as the quality of the immune response. Vaccine antigens must mimic the natural pathogen's first signal, which

is recognized by innate immune cells' pattern recognition receptors (PRRs). Certain vaccine antigens bind to APCs using toll-like receptors (TLRs) (Barton & Medzhitov, 2002). Activating an inflammatory cascade that results in APC migration to the draining lymph node and cytokine

production. This activates other immune cells, including naïve CD4+ T cells, by presenting antigenic peptides to MHC-II molecules. B cells, which also function as APCs, get activated when exposed to the vaccination antigen and develop into plasmocytes that produce low affinity IgM antibodies. B cells are activated in the presence of CD4+ T cells, causing them to create high-affinity IgG or IgA antibodies as well as memory cells via genetic changes. This emphasizes the significance of activating CD4+ T cells and APCs, particularly dendritic cells, and the use of adjuvants (Zepp, 2016).

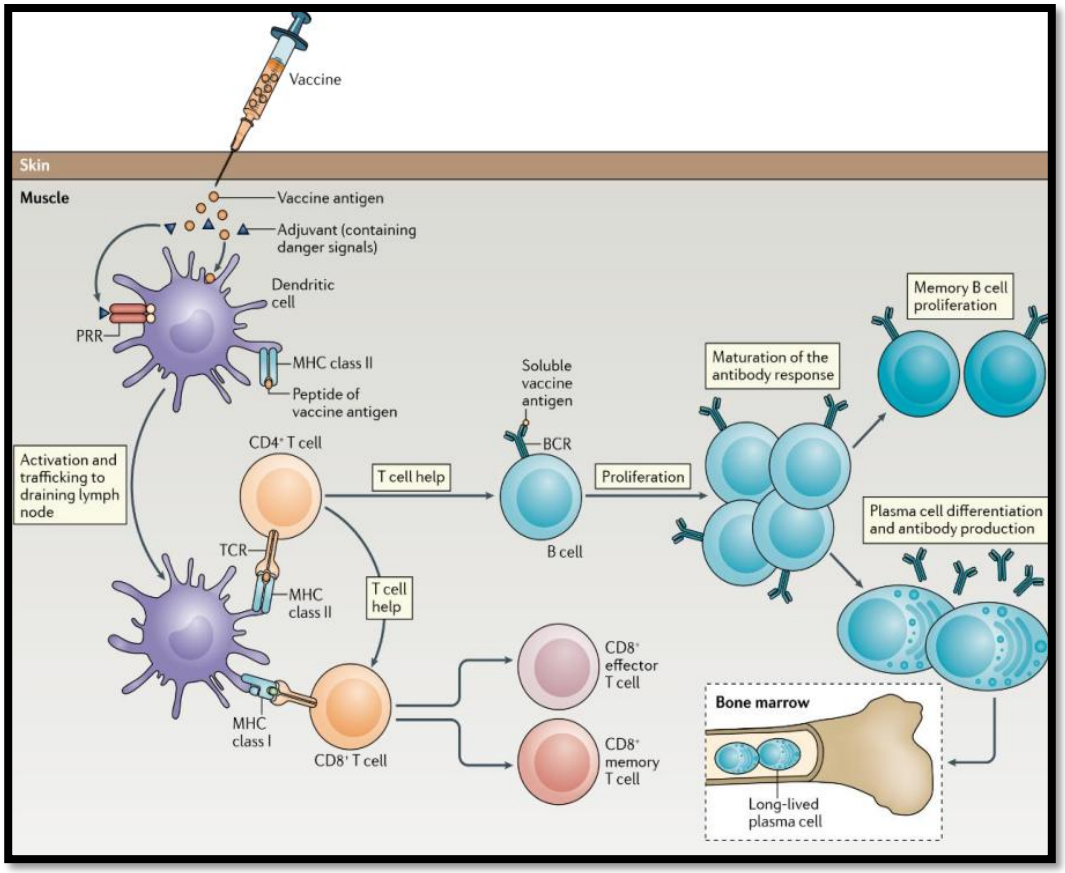


Figure 4: The generation of an immune response to a vaccine (Pollard & Bijker, 2021).

Chapter III:

Cisplatin and Chemotherapy

Chapter III: Cisplatin and Chemotherapy

1. Overview of chemotherapy

Chemotherapy, either alone or in conjunction with surgery, radiotherapy, or targeted therapies, continues to be one of the primary cancer treatment modalities (Longley, D. B., & Johnston, P. G., 2005). Cancer remains a leading cause of mortality globally and a major health burden, with chemotherapy often serving as the primary therapeutic option for patients with advanced-stage disease, partially compensating for the limitations of non-curative treatments. However, the emergence of chemotherapy resistance significantly limits its efficacy, posing a major clinical challenge. Moreover, heterogeneity of resistance mechanisms across cancer types complicates the development of universally effective diagnostic and therapeutic approaches. (Gu, Y., et al.,2025).

Due to its lack of selectivity, conventional chemotherapy can harm healthy tissues and cause serious adverse effects (Pérez-Herrero, E., & Fernández-Medarde, A.,2021). On the other hand,Cytotoxic chemotherapy mostly causes cell death by obstructing DNA replication or cell division, especially in rapidly growing cells (Chabner, B. A., & Roberts, T. G.,2005).

2. Cisplatin: Definition and mechanism of action

2.1. Definition of Cisplatin

Cisplatin is one of the most effective chemotherapeutic drugs used against various types of cancers; nevertheless, its therapeutic use is frequently limited by severe immunosuppressive side effects (Elmorsy et al., 2024). Cisplatin-induced immunosuppression is mainly associated with excessive generation of reactive oxygen species (ROS), oxidative stress, and DNA damage that promote apoptosis of immune cells, particularly lymphocytes (Dai et al., 2024). These alterations negatively affect both innate and adaptive immune responses, resulting in impaired immune cell proliferation and dysregulation of cytokine secretion (Elmorsy et al., 2024). In addition, cisplatin may induce myelosuppression characterized by a reduction in white blood cells, which consequently increases susceptibility to infections and weakens the immune defense system (Dai et al., 2024). Recent studies have also reported that mitochondrial dysfunction and activation of inflammatory pathways play a significant role in cisplatin-mediated immune toxicity (Elmorsy et al., 2024). Therefore, understanding the molecular mechanisms involved in cisplatin-induced immunosuppression is important for developing protective approaches capable of minimizing its adverse effects while maintaining its antitumor activity (Dai et al., 2024).

2.2. mechanism of action

- Cisplatin binds to DNA and forms intra- and inter-strand crosslinks, which ultimately trigger apoptosis, thereby exerting its cytotoxic effects (Pérez-Herrero, E., & Fernández-Medarde, A., 2021).
- Once inside the cell, cisplatin undergoes aquation, which increases its reactivity with nucleophilic sites on DNA by substituting water molecules for chloride ligands (Longley, D. B., & Johnston, P. G., 2005).
- Many signal transduction pathways, including those leading to cell cycle arrest and programmed cell death, are activated by the DNA damage caused by cisplatin (Chabner, B. A., & Roberts, T. G., 2005).
- The lack of selectivity in conventional chemotherapy causes harm to healthy tissues and the emergence of serious side effects (Pérez-Herrero, E., & Fernández-Medarde, A., 2021).

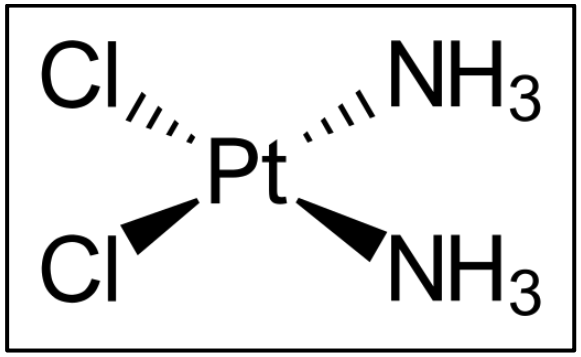


Figure 5: Chemical structure of cisplatin (National Toxicology Program.,2021).

3. Pharmacological properties of cisplatin

Cisplatin is a chemotherapeutic drug that has been used in the treatment of various types of human cancers such as ovarian, lung, head and neck, testicular and bladder. Cisplatin has demonstrated efficacy against various types of cancers such as germ cell tumors, sarcomas, carcinomas as well as lymphomas (Aldossary, S. A.,2019).

3.1. Cisplatin-Induced Immunosuppression

Cisplatin is a first-generation platinum-based chemotherapeutic agent that has been extensively used for the treatment of numerous solid malignancies, including lung, ovarian, bladder, testicular, and head and neck cancers (Nagai & Kim, 2017). Its antitumor activity mainly results from the formation of DNA adducts and crosslinks that disrupt DNA replication and transcription, ultimately triggering cell cycle arrest and apoptosis in rapidly proliferating cancer cells (Wang & Lippard, 2005). In addition to its direct interaction with nuclear DNA, recent studies demonstrated that cisplatin can induce mitochondrial

dysfunction, oxidative stress, and excessive production of reactive oxygen species (ROS), which further contribute to its cytotoxic effects (Peres & da Cunha, 2022). Although cisplatin remains one of the most effective anticancer drugs, its clinical application is frequently associated with severe side effects such as nephrotoxicity, hepatotoxicity, neurotoxicity, ototoxicity, and immunosuppression, mainly due to damage in normal tissues (Manohar & Leung, 2018). Recent advances in cancer research have focused on developing novel nanotechnology-based delivery systems and antioxidant therapeutic strategies to reduce cisplatin toxicity while maintaining its potent anticancer efficacy (Yuan et al., 2024).

3.2. Cisplatin-Induced Toxicity

Cisplatin is an effective anticancer drug widely used in chemotherapy, but its clinical application is strongly limited by its toxicity in several organs, mainly due to oxidative stress and cellular damage (Oun et al., 2018). In the kidneys, cisplatin accumulates in proximal tubular epithelial cells, leading to acute kidney injury characterized by inflammation, tubular necrosis, reduced filtration capacity, and vascular dysfunction (Perše & Večerić-Haler, 2023). In the nervous system, cisplatin induces peripheral neuropathy through axonal degeneration, synaptic dysfunction, and neuroinflammation associated with increased oxidative stress and mitochondrial impairment (Staff et al., 2022). In the cardiovascular system, cisplatin can cause myocardial injury and endothelial dysfunction, which increases the risk of long-term cardiovascular complications due to reactive oxygen species–mediated cellular damage (Herradón et al., 2021). In the liver, cisplatin induces hepatotoxicity manifested by hepatocellular injury, inflammatory responses, and altered liver enzyme activity, mainly driven by lipid peroxidation and oxidative stress (Almeer & Alarifi, 2022). In the auditory system, cisplatin leads to irreversible hearing loss by damaging cochlear hair cells, where oxidative stress and depletion of antioxidant defenses play a central role (Sheth et al., 2021). Overall, oxidative stress is the main mechanism behind cisplatin-induced multi-organ toxicity, as it increases reactive oxygen species (ROS) while reducing antioxidant enzymes such as glutathione (GSH), superoxide dismutase (SOD), and catalase, resulting in mitochondrial dysfunction, DNA damage, and apoptosis across different tissues (Zhang et al., 2023).

Chapter IV:

Nanotechnology and Nanoparticles

Chapter IV: Nanotechnology and Nanoparticles

1. Nanotechnology

Nanotechnology involves the production of particles that are between 1 to 100 nm in size by using various synthesis methods, as well as modifying their structure and size. The use of nanoparticles has become increasingly prevalent across a variety of fields, including molecular biology, physics, organic and inorganic chemistry, medicine, and material science. When particles are reduced to nano-size, they exhibit unique and improved properties, such as particle size distribution and morphology, that are not seen in larger particles of bulk material (Jamkhande, P. G., Ghule, N. W., Bamer, A. H., & Kalaskar, M. G. ,2019).

2. Nanomedicine

Nanomedicine is an emerging interdisciplinary field that applies nanotechnology in the diagnosis, treatment, monitoring, and prevention of diseases through the use of nanoscale materials and devices (Patra et al., 2018). It mainly involves the development of nanoparticles, nanocarriers, nanosensors, and nano-based drug delivery systems designed to improve therapeutic efficacy while minimizing the adverse effects associated with conventional therapies (Ventola, 2017). Due to their nanoscale size and high surface area, nanoparticles can efficiently cross biological barriers and preferentially accumulate in diseased tissues, particularly tumors, thereby enhancing drug bioavailability and controlled release (Mitchell et al., 2021).

Nanomedicines are extensively investigated for cancer therapy due to their ability to improve the selective delivery of anticancer drugs to tumor sites while reducing accumulation in healthy tissues, thus optimizing the balance between efficacy and toxicity (Lammers, 2024). More than 20 nanomedicine-based formulations have already been approved for clinical use, while hundreds of others are still under preclinical and clinical development. Despite these advances, several biological and physiological barriers continue to limit efficient tumor targeting and clinical translation, representing major challenges in the field of cancer nanomedicine (Lammers, Ferrari & Nieberler, 2024).

Recent advances in nanomedicine also include biomimetic nanoparticles, protein-conjugated nanocarriers, and stimuli-responsive systems designed to achieve more precise and personalized cancer therapies (Mitchell et al., 2021).

3. Nanoparticles

Spherical particles called nanoparticles are composed of either natural or artificial polymers and can range in size from 10 to 500 nm. Due to their high surface area-to-volume ratio and spherical shape,

these particles have a variety of potential applications. (Rahim, M., Rizvi, S. M., Iram, S., Khan, S., Bagga, P. S., & Khan, M. S., 2018).

NPs can be categorized into organic, inorganic, and carbon-based particles on the nanometric scale that exhibit improved properties in comparison to their larger counterparts based on the material. (Kumari, S., & Sarkar, L., 2021)

3.1. Organic nanoparticales

Liposomes, polymersomes, polymer constructions, and micelles are examples of organic nanoparticles that have been extensively studied and used in imaging or medication and gene delivery methods. Organic Nps are frequently employed in biomedical sectors and are generally biodegradable and biocompatible.

3.2. Inorganic nanoparticales

Because of their distinct material- and size-dependent physicochemical features, inorganic nanoparticles have also drawn the interest of researchers in recent years.typically comprise of metals and metal oxides, and they have drawn significant funding from both commercial and scientific sources. (Khalid, K. ,2020).

4. Copper Oxide Nanoparticles (CuNPs) :

Copper oxide nanoparticles (CuNPs) are a class of metal oxide nanoparticles that have gained considerable attention due to their low cost, ease of preparation, and remarkable physicochemical properties. These nanoparticles exhibit high surface area, excellent catalytic activity, and significant antimicrobial potential, which make them attractive for various biomedical and industrial applications .(Rashad, M. M., Elsayed, E. M., & Al-Kotb, M. S., 2021).

5. Green Synthesize of Copper Nanoparticles:

In recent years, nanoparticle synthesis has witnessed a remarkable shift toward sustainable and environmentally friendly approaches. Conventional nanoparticle synthesis methods frequently involve using hazardous chemicals and high-energy processes, raising environmental concerns, and producing toxic by-products. On the other hand, green synthesis methods provide a viable solution by utilizing bio-based materials .

(Osman, A. I., et al., 2024)

5.1. Synthesis of CuNPs using Plant Extract

The green synthesis of copper nanoparticles (CuNPs) using plant extracts is considered an eco-friendly, cost-effective, and sustainable method for nanoparticle production (Ibrahim et al., 2022).

Plant extracts contain bioactive compounds such as polyphenols, flavonoids, and proteins that act as natural reducing and stabilizing agents during nanoparticle formation (Sajjad et al., 2023).

These phytochemicals reduce Cu^{2+} ions into elemental copper nanoparticles while preventing aggregation and improving stability (Khan et al., 2024).

This approach is widely preferred over chemical methods due to its simplicity, safety, and environmental compatibility (Ibrahim et al., 2022).

5.2. Synthesis of CuNPs using Microorganisms

Microbial synthesis of copper nanoparticles involves bacteria, fungi, and yeast that act as biological factories for nanoparticle production (Iravani et al., 2020).

These microorganisms reduce metal ions through enzymatic and metabolic activities, often involving reductase enzymes (Singh et al., 2021).

The process can occur intracellularly or extracellularly depending on the microbial species and reaction conditions (Abdel-Raouf et al., 2023).

This method is advantageous due to its biocompatibility, scalability, and ability to control nanoparticle size and morphology (Iravani et al., 2020).

5.3. Synthesis of CuNPs using Polymers

Polymer-assisted synthesis of copper nanoparticles is widely used to control particle size and prevent aggregation (Zhang et al., 2022).

Polymers such as PEG, PVA, and chitosan act as both stabilizing and capping agents during nanoparticle formation (Li et al., 2023).

These polymers regulate nucleation and growth processes, leading to uniform and stable nanoparticles (Wang et al., 2024).

Additionally, polymer-coated CuNPs exhibit improved biocompatibility and are suitable for biomedical applications including drug delivery systems (Zhang et al., 2022).

6. Biomedical Application of CuNPs

Copper nanoparticles (CuNPs) have attracted considerable attention in biomedical applications due to their unique physicochemical properties, including high surface reactivity and strong redox activity (Rafique et al., 2022).

CuNPs exhibit strong antimicrobial activity against a wide range of bacteria, fungi, and viruses through the generation of reactive oxygen species (ROS) and disruption of microbial cell membranes (González et al., 2023).

These nanoparticles can also interfere with microbial DNA and protein synthesis, leading to irreversible cellular damage and cell death (Khan et al., 2024).

In cancer therapy, CuNPs have shown promising anticancer activity by inducing oxidative stress, mitochondrial dysfunction, and apoptosis in malignant cells (Zhang et al., 2023).

Moreover, CuNPs can be functionalized with biomolecules or polymers to improve their stability, biocompatibility, and targeted delivery to tumor tissues (Li et al., 2024).

Recent studies have also highlighted their potential in drug delivery systems, wound healing, and biosensing applications, making them versatile tools in nanomedicine (Patel et al., 2022).

Nanoparticles can be engineered to deliver antigens and adjuvants to antigen-presenting cells, thereby enhancing immune responses and improving vaccine efficacy (Irvine, D. J., & Read, B. J., 2020). In order to improve anti-tumor immune responses, immunomodulatory substances such as cytokines, antigens, and nucleic acids are being delivered using nanoparticles in cancer immunotherapy. (Riley, R. S., June, C. H., Langer, R., & Mitchell, M. J.,2019)

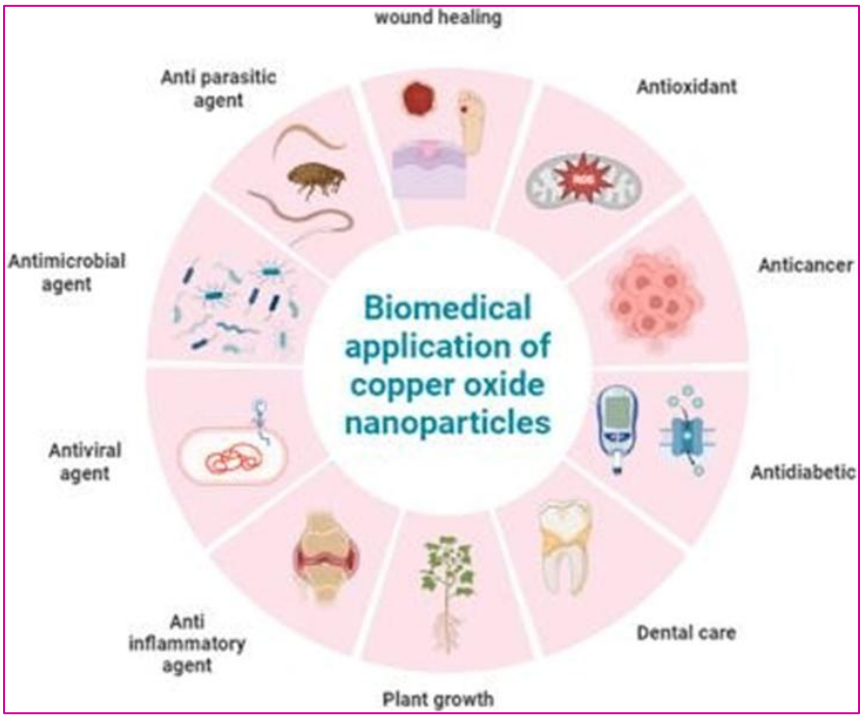


Figure 6 : Biomedical Applications of CuNPs (Devaraji, M.,et al 2024)

Second Part

Experimental Part

Chapter I:

Material and Methods

I.Material

1. Biological materials

1.1. Reagents and products:

Copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), human serum albumin (HSA), sodium hydroxide (NaOH), phosphate-buffered saline (PBS), trichloroacetic acid (TCA), dipotassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), ethanol, ethylenediaminetetraacetic acid (EDTA), physiological solution (NaCl 0.9%), distilled water, potassium ferricyanide, ferric chloride ($FeCl_3$), ascorbic acid, bovine serum albumin (BSA), diclofenac sodium, concentrated hydrochloric acid (HCl), thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), salicylic acid, Tris buffer, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), nitro blue tetrazolium (NBT), riboflavin, tris(hydroxymethyl)aminomethane (Tris), and sodium chloride (NaCl) were used in this study.

1.2. Breast Cancer Tissue Samples

Breast cancer tissue samples were obtained directly from female patients diagnosed with breast cancer at Cancer Control Center in El-Oued, Algeria – Chahid Rezgui Bashir.

2. Animals

2.1.Animals care:

In this study, twenty females of Albino Wistar rats at the age of 8–10 weeks old, weighting 130.52±2.61, obtained from Pasteur Institute in Algiers. Rats were given a free access to their standard diet and tap water during the study. The animals were carried under the same conditions of and an ambient temperature with a 12-hour light/dark cycle. All experimental procedures employed, as well as rats' care and handling, were in accordance with guidelines provided by the local ethics. After a 14-day adaptation period, the animals were randomly divided into experimental groups. The experiment was conducted over a period of one month

2.2.Experimental design:

On the same day, the control group received an intraperitoneal injection of physiological saline (0.9% NaCl), while the remaining groups were injected intraperitoneally with cisplatin at a single dose of 5 mg/kg body weight to induce oxidative stress, as previously reported by Majd, N. E. (2023).

One week after cisplatin administration, the treatment protocols were initiated. Groups I and II received physiological saline only via intraperitoneal injection. Group III was treated intraperitoneally with CuONPs-BCC at a dose of 0.5 mg/kg/day, according to the protocol described by Abdel-Kader, M.

(2025). Group IV received breast cancer tissue homogenate (0.1%) through intraperitoneal injection, following the method of Abd El-Fattah, A. (2017). This treatment procedure was repeated once weekly for three consecutive weeks.

The experimental groups were designed as follows:

Group I (Control): Rats received intraperitoneal injections of physiological water throughout the experimental period.

Group II (Cisplatin): Rats received a single intraperitoneal injection of cisplatin (5 mg/kg body weight). One week later, they were injected with physiological water.

Group III (CuONPs-BCC): Rats received cisplatin (5 mg/kg, intraperitoneally). One week later, they were treated with CuONPs-BCC (0.5 mg/kg/day, intraperitoneally).

Group IV (Homogenate): Rats received cisplatin (5 mg/kg, intraperitoneally). After one week, they were administered breast cancer tissue homogenate (0.1%, intraperitoneally).

The CuONPs-BCC suspension was prepared using an ultrasonic technique for 30 min at 40 °C before each administration. Body weights were recorded weekly throughout the experimental period.

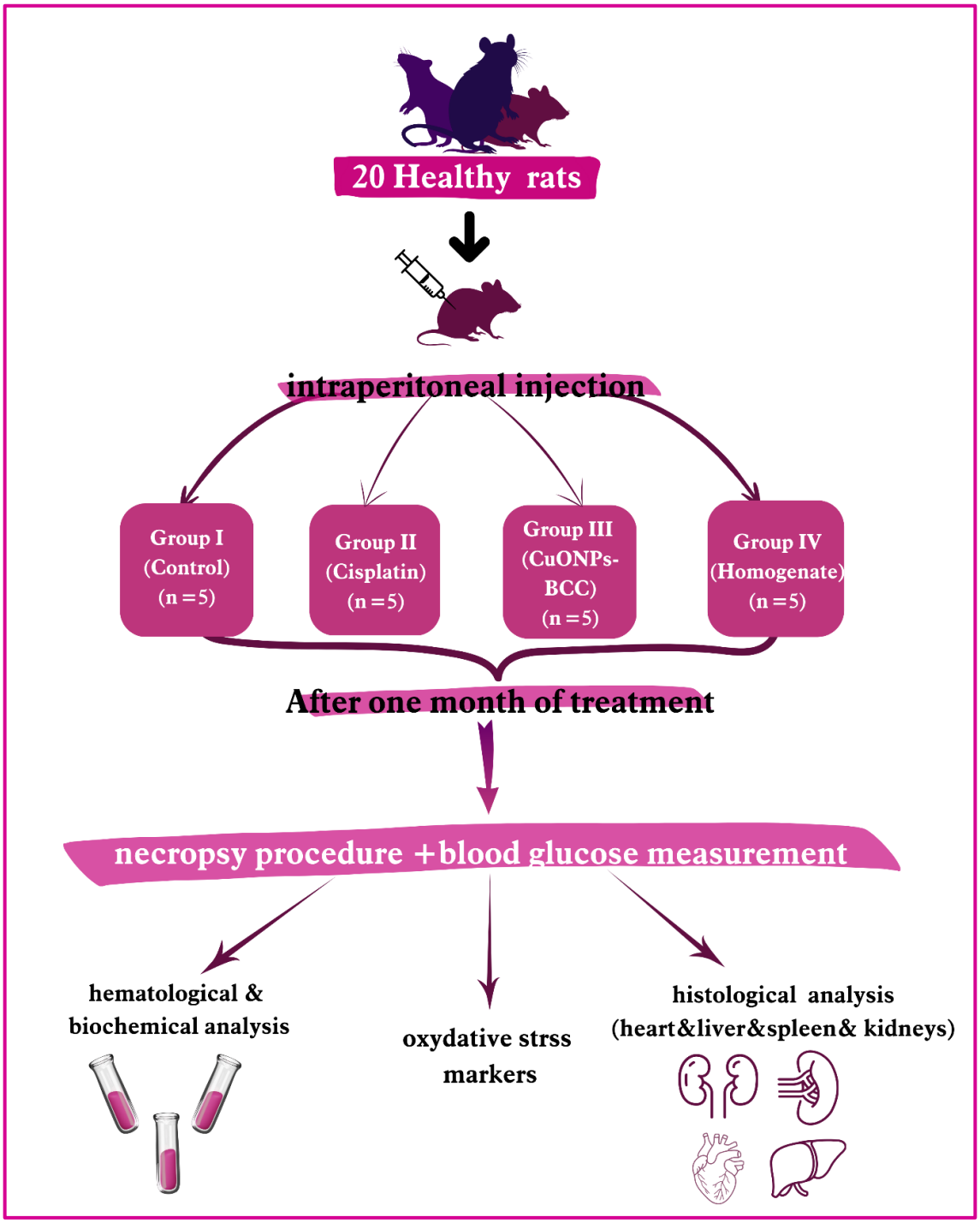


Figure 7: Experimental design of the study.

2.3. Sacrifice, blood sampling and tissue collection

One week after vaccination, the rats were sacrificed following a 16-hour fasting period. under slight anesthesia by chloroform (94%) by inhalation. During euthanasia, blood samples were collected from the animals and placed in EDTA tubes for hematological analysis and heparin tubes for biochemical analysis. The serum was obtained by centrifuging for 5 minutes at 1500 rpm, and blood sugar was measured by a glucometer. The weights of the organs were recorded, washed with saline (Nacl 0.9%),

and frozen at -20 °C until use. The spleen, the heart, the kidneys and the liver tissues are fixed in 10% formaldehyde for histological analysis.

II. Methods

1. In-vitro study

1.1. Green Synthesis of Copper Oxide Nanoparticles Using Human Serum Albumin (CuONPs)

In order to synthesize the Copper nanoparticles, CuSO4 (2 mg as $Cu^{2+)}$ 5 mg and HSA 10 mg were separately dissolved in 10 mL of double distilled water (DDW). After preparation, the CuSO4 and HSA solution were mixed, and then 400 µL of 1 M NaOH was added to the mixture to adjust the pH to 9. The synthesis reaction was continued for 30 min at room temperature with stirring at 1000 rpm. The final CuONPs suspension was concentrated to 1 mL and stored in a refrigerator (4 °C) before use (Amatya et al., 2023).

1.2.Homogenate preparation

Two grams of tissue were weighed and homogenized in 5 mL of buffer solution (PBS, pH 6.8), supplemented with 0.5 mL of 20% trichloroacetic acid (TCA). The homogenate was centrifuged at 3500 rpm for 10 minutes to separate supernatant I (Solution A) from pellet I. Pellet I was subsequently treated with 2 mL of 0.1 N NaOH, and a second centrifugation at 3500 rpm for 10 minutes yielded supernatant II (Solution B) (Derouiche, S.,2017).

1.3. Synthesis of bio-conjugated CuONPs with snail cell “Breast Cancer Cells” (CuONPs-BCC)

5mL of breast cancer cells was kept in a 50 mL tube with 300 mg of CuNPs. Using distilled water, fully mixed, and rotated at a steady 500 rpm. The samples were centrifuged after four hours, and the resulting Copper-homogenate nanocomposite was then rinsed twice with distilled water and stored in a refrigerator at 4°C for additional research (Amatya, R.,2023).

1.4. Characterization of Copper Oxide-Nanocomposites

The characterization of Copper was identified by Ultraviolet-visible spectroscopy (UV-VIS), Fourier transform infrared spectroscopy (FTIR), Scanning electron microscope (SEM), energy dispersive X-ray (EDX) and X-ray diffraction (XRD). The analysis were done by direct reading through JENWEY, Cary 630 FTIR and Phenom Prox, respectively.

For XRD analysis, the crystallite size was estimated using the Debye–Scherrer equation, which relates the broadening of diffraction peaks to the size of coherently diffracting crystalline domains (Fatimah et al., 2021; He et al., 2018). The equation is expressed as:

$$D = \frac{K}{\beta \cos(\theta)}$$

where D is the crystallite size, K is the shape factor (typically 0.9), λ is the X-ray wavelength (0.15406 nm for Cu Kα radiation), β is the full width at half maximum (FWHM) of the diffraction peak expressed in radians, and θ is the Bragg diffraction angle. (Monshi et al., 2012; Mongkolsuttirat & Buajarern, 2021).

1.5. Biological activities

- **Antioxidant activity (Ferric Reducing Ability Test "FRAP")**

FRAP assay assess the antioxidant activity of sample through reduction of ferric ion (Fe3+) to ferrous ion (Fe2+) by antioxidants present in sample. According to Oyaizu (1986), 1000 µL of different concentrations (200, 175, 150, 125, 100, 75, 50, 25, 10 and 5 µg/mL) of CuONPs-BCC was mixed with 2500 µL of phosphate buffer solution (pH 6.6; 0.2 M), then 2500 µL of potassium ferricyanide (1%) was added. The mixtures solutions were incubated in water bath for 20 minutes at 50°C, the reaction was stopped by adding 2500 µL of trichloroacetic acid (10%). The mixtures reactions were centrifuged at 3000 rpm for 10 min, then 2500 µL of the supernatant, 2500 µL of distilled water, and 500 µL of ferric chloride (0.1 %) were mixed. The sample absorbance (A1) was measured at λ= 700 nm. Various concentrations of ascorbic acid (standard) were performed under the same experimental processed conditions of the samples. The negative control was a blank (A0). The results express using IC50 values which was estimated using the regression line equation against different concentrations of samples using FRAP values which calculated accordin to Yazdani et al. (2019):

$$\text{FRAP (\%)} = (A1\text{-}A0)/A1 \times 100$$

- **Anti-inflammatory activities (Inhibition of Protein Denaturation Ability Test)**

In vitro anti-inflammatory activity of CuONPs-BCC were studied using inhibition of protein denaturation assay according to Vennila et al. (2018). Various concentrations (10–100 µg/mL) of CuONPs-BCC was mixed with bovin serum albumin solution (1%), the mixture solutions were incubated during 30 min at ambient temperature. After that, the reaction solution's pH was adjusted to 2 using a few drops of concentrated hydrochloric acid solution; then, the mixture solutions were exposed to heating in 72 °C during 30 min; after the incubation, the tubes were cooled for 10 min in ice bath. Finally, the sample absorbance (A1) was measured at λ= 660 nm. The diclofenac (Standard) were

exposed to the same experimental condition of the sample. The blank was used as negative control (A0). The results were expressed as IC50 which calculated using the line regression of inhibition percentage:

$$\text{Inhibition percentage (\%)} = (A0-A1)/A0\times100$$

❖ **Anti-hemolytic ability test**

Anti-hemolysis test was performed according to Vinjamuri et al. (2015) which assess the protective capacity of bioactive compounds in the sample against red blood cell (RBC) membrane lysis induced by 1X PBS. 5mL of blood was obtained from healthy volunteers was placed into EDTA tube to stop its coagulation, then the blood centrifuged at 4°C for 10 min at 1000 tour/min. the pipette was used with the utmost care to remove totally the plasma and white buffy layer. The erythrocytes were then washed three times with 1X PBS (0.1 M; pH 7.4) using centrifugation method for 5 minutes. Erythrocytes that had been washed were kept at 4°C and used within 6 hours for the test. 50 µL of diluted erythrocytes suspension 10% were mixed with 100 µL of various concentration (10-100 µg/mL) of CuONPs, CuONPs-BCC, a positive control was 100 µL of different concentrations of diclofenac and a negative control was 100 µL of 1XPBS. After that, the reaction solutions were incubated during one hour at 37°C in water bath. The volume of reaction mixtures was completed to 1 mL by adding 1X PBS, then centrifuged at 300 rpm for 3 minutes. The hemoglobin amount in the supernatant of samples (A0) were subsequently determined using a spectrophotometer (Jenwey) at λ= 540 nm. The results were expressed by IC50 which were calculated using the percentage of hemolysis as following:

$$\text{Hemolysis inhibition (\%)} = (A0-A1)/A0\times100$$

2. In-vivo study

2.1. Acute Toxicity Test

In this experimental study, The acute toxicity of CuONPs-BCC was evaluated according to the method described by Lorke (1983). Six females of albino Wistar rats (8–10 weeks old, 159.67± 8.09) randomly assigned to three groups (n = 2). After 12 h of fasting; the rats of each group were:

Group 1 served as the untreated control.

Group 2 received a single intraperitoneal injection of CuONPs-BCC at 2.5 $mg.kg^{-1}$ of body weight

Group 3 received the same formulation at 5 $mg.kg^{-1}$ of body weight.

Animals were observed for 14 days following administration and were assessed daily, in comparison with the control group, for mortality, ocular appearance, sleep patterns, locomotor activity, and the presence of diarrhea, in accordance with the predefined clinical criteria described in the laboratory protocol. **(Lorke ,1983).**

2.2. Hematological parameter analysis

Hematological analyses were conducted using the XN-330 analyzer (Sysmex) in order to determine the leukocyte, erythrocyte, and platelet parameters.

2.3.Biochemical parameters

Determinations of biochemical parameters: Fasting blood glucose, Relative weight , CRP and IgG .

2.4. Oxidative stress tests

2.4.1. Determination of Malondialdehyde (MDA) level

❖ **Principle**

The common method for the assessment of MDA level is the thiobarbituric acid (TBA) assay, where MDA forms a complex with two molecules of 2-thiobarbituric acid (TBA) in the presence of acidic medium and heat. A change in the color of the solution to a pink color is an indication of the presence of MDA A (Yagi, 1976).

❖ **Reagent**

375 mg of TBA, 20 g of TCA, 0.01 g of BHT, 25 ml of 1N HCL, and 50 ml of distilled water were mixed in a beaker. The solution obtained was heated to 40 °C in a water bath until the TBA was completely dissolved, then transferred to a 100 mL flask, and the volume was filled up with distilled water.

❖ **Procedure**

Pipette into the glass vise test tubes 200 µl of sample and 800 µl of TBA reagent and close tightly. Heat the mixture in a water bath at 100 °C for 15 minutes. Then cool in a coldwater bath for 30 minutes, leaving the tubes open to allow the gases formed during the reaction

to be removed. Centrifuge at 3000 rpm for 5 minutes and read the absorbance of the supernatant at 532 nm using a spectrophotometer.

❖ **Expression of results**

The concentration of TBARS was determined using the molecular extinction coefficient of MDA (å = 1.53 105 M-1 cm-1). The results were expressed in µmol/1.

MDA (µmol/mg of t) = (OD sample /1.53 x 10^5) / mg of Tissue

2.4.2. Determination of reduced glutathione (GSH) level

❖ **Principal**

The level of reduced glutathione is determined according to Weckbecker & Cory, (1988). By measuring the optical density results from the formation of 2-nitro-5- mercocapturic acid (TNB) from the reduction of dithio-bis-2-nitrobenzoic acid (DTNB), which is called Ellman reagent with SH groups exist in GSH briefly

❖ **Procedure**

800 µl of homogenate samples are added to 200 µl of salicylic acid (0.25%). Then centrifuged at 1000 rpm for 5 min.

Take 500 ml of supernatant and mix it with 1000 µl of tris buffer solution (tris 0.4 mol, NaCl 0.02 mol; ph = 8.9) and 25 µl of DTNB (0.01 mol/L).

Read the absorbance at λ = 412 nm after 5 minutes of incubation.

❖ **Expression of results**

$$GSH(nmol/mg\ of\ prot) = \frac{(OD \times 1 \times 1.525)}{13133 \times 0.8 \times 0.5 \times mg\ of\ prot} \times d$$

2.4.3. Determination of Super Oxide Dismutase (SOD) activity

❖ **Principle**

This test is based on the inhibition of NBT reduction by SOD. NBT is reduced by the superoxide anion O2-, and it is known that SOD neutralizes O2-, which inhibits the reduction of NBT (Beauchamp & Fridovich, 1971).

❖ **Procedure**

50 µL of sample was introduced into test tubes and mixed with 1000 µL (0,1 mM, 13 mM), 1800µL phosphate buffer solution (50 mM; pH 7.8) and 100 µL of NBT (75 µM). The tubes were incubated for 5 min at 25°C. 50 µL of riboflavin (2 µM) were then added and incubated in a light box for 20 min. The optic density was read at 560 nm. The SOD activity was expressed on Unit/mg of tissue.

❖ **Expression of results**

Inhibition percentage of NBT reduction by SOD as follows:

$$IP\% = \frac{OD\ blanc - OD\ sample}{OD\ blanc} \times 100$$

50% (IP) =1U of SOD

2.5. Histological study

Histological sections were done following the method of Ishfaq et al., (2019), with a slight modification. 10% buffered formalin was used for fixing spleen, liver, heart, kidenys samples, followed by dehydration with graded ethanol. The samples were processed for paraffin wax and cut into sections (5 µm thickness). After that, the sections were mounted on slides, stained with hematoxylin and eosin dye, and observed under a light microscope (10X and 40X magnifications).

2.6. Statistical analysis

The results obtained are expressed as the mean ± standard error of the mean (Mean ± SEM). The analysis of the data was carried out by application of the Student's T test. All data in this study were examined by Excel and Minitab software. $p < 0.05$ indicates statistically significant difference.

Chapter II:

Results and Discussion

I. Results

1. In-vitro assays of copper oxide nanoparticles

1.1. Characterization of CuONPs

1.1.1. UV–Vis spectroscopy

Figure 8 (A) showed UV-visible spectrum of CuONPs in the range of 200–400 nm, which revealed that the maximum absorption peak for CuONPs is located at around 240 nm.

In addition, the spectrum of CuONPs-BCC (B) showed the maximum absorption peak at approximately 250 nm, which may be related to the protein components present in the formulation. The shift in peak position from 250 nm to 240 nm confirms the formation of copper nanoparticles.

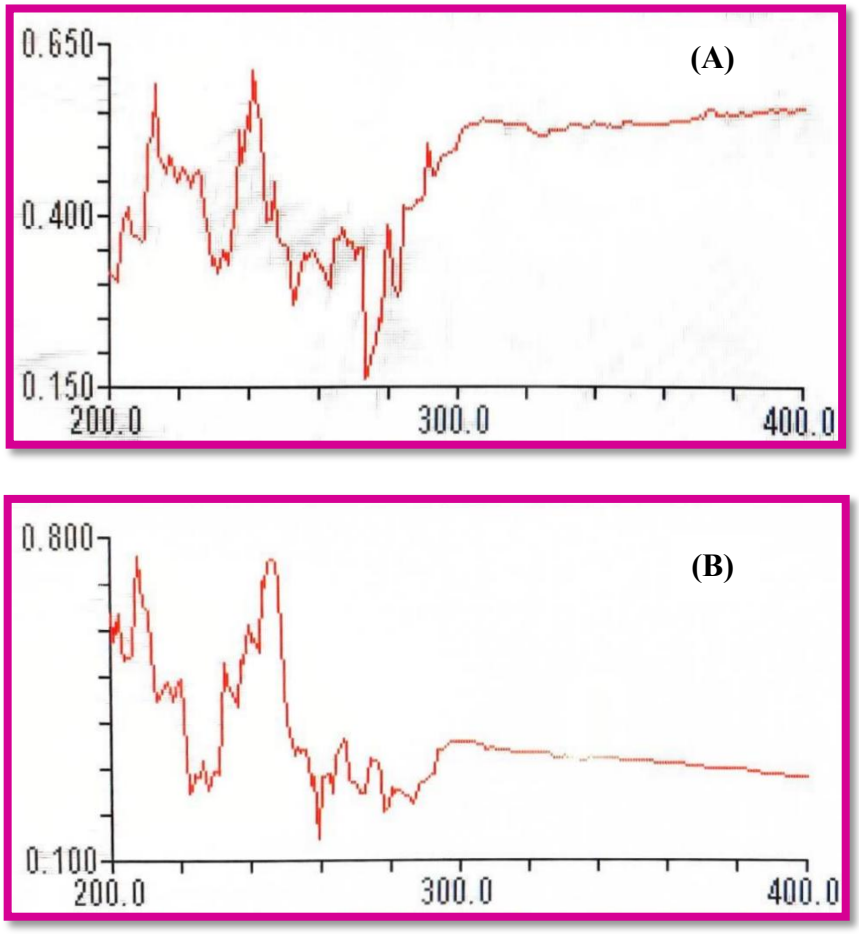


Figure 8: *UV-Vis spectrum of* CuONPs (A) *and* CuONPs-BCC (B)

1.1.2. FT-IR analysis

Figure 9 showed the FTIR spectra of CuONPs (A) and CuONPs-BCC (B) recorded in the range of 400–4000 cm^{-1}. It was observed that a characteristic absorption peak appeared in the region between 400–600 cm^{-1}. confirming the formation of copper nanoparticles. The difference between the composition of CuONPs-BCC and CuONPs was evident from the appearance of different peaks for each spectrum.

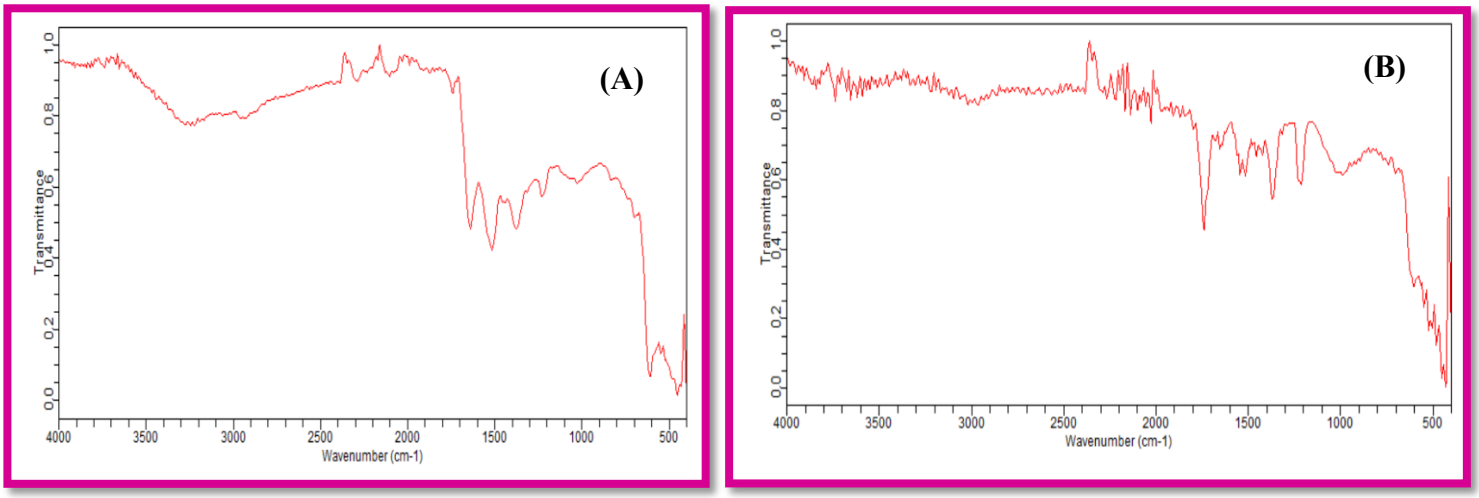


Figure 9: FT-IR spectra *of* CuONPs (A) *and* CuONPs-BCC (B)

1.1.3.XRD studies

❖ **CuONPs**

The synthesized CuONPs XRD pattern exhibits diffraction peaks at 2θ values of 28.42°, 29.56°, 36.42°, 42.22°, 61.58°, and 73.48°. The peaks are assigned to (110), (111), (200), (220) and (311) crystallographic planes, respectively, confirming formation of cubic copper(I) oxide (Cu_2O) with cuprite-type structure. The diffraction peaks correspond to the standard JCPDS card No. 05-0667 confirming the formation of Cu_2O nanoparticles (Norouzi et al., 2021; Zeghdi et al., 2024). The most intense peak at around 36.42° corresponding to (111) plane shows preferred orientation along this direction which is often observed in Cu2O nanostructures due to anisotropic growth at nanoscale (Agarwal et al., 2020) .The low angle reflection shifts slightly (around 28.42-29.56°) from its normal position (~29.6°) which may be due to lattice strain, peak broadening due to size or defects like oxygen vacancies. These effects are well known in the case of nanocrystalline materials and confirm the non-bulk character of the synthesized Cu_2O (Agarwal et al., 2020; Zeghdi et al., 2024). The crystallite size calculated using the Debye–Scherrer equation (considering the strongest (111) peak at 36.42°) and an FWHM of 1.04° is ~8.0 nm, confirming the nanocrystalline nature of the material. The small crystallite

size indicates a high surface to volume ratio which is beneficial to enhance the surface reactivity, adsorption capacity and photocatalytic performance (Norouzi et al., 2021; Zeghdi et al., 2024). **(Figure 10)**

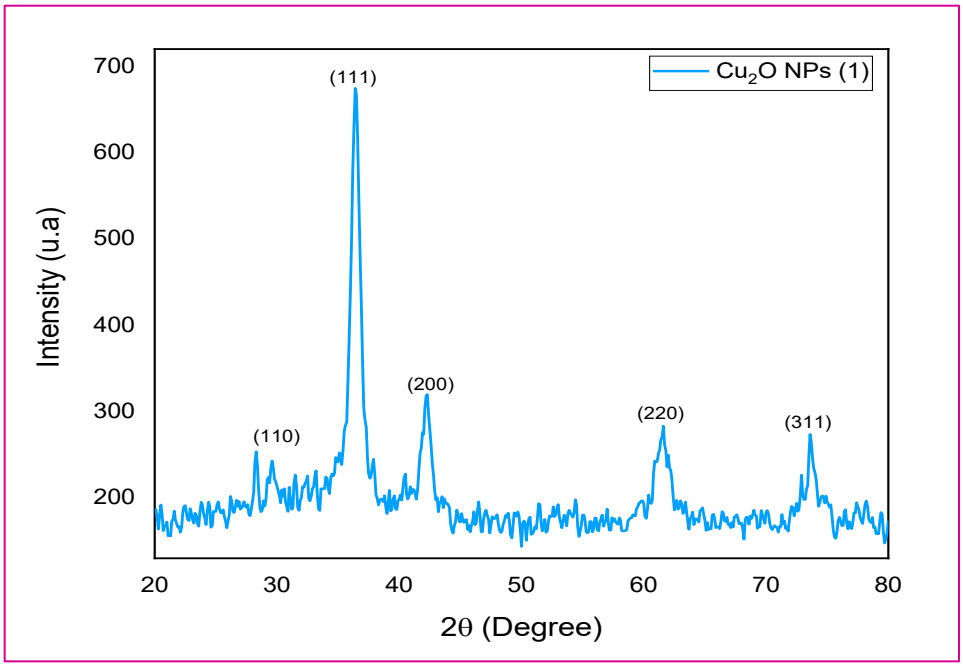


Figure 10: XRD pattern of CuONPs

❖ **CuONPs-BCC**

The X-ray diffraction (XRD) pattern of the synthesized CuONPs-BCC nanoparticles shows intense diffraction peaks at 2θ values of 30.32°, 36.66°, 42.76°, 61.81° and 73.63°. They are indexed to the (110), (111), (200), (220) and (311) crystallographic planes respectively which correspond to the cubic copper(I) oxide (Cu_2O) with a cuprite structure. The observed diffraction peaks are well matched with the standard JCPDS card No. 05-0667, confirming the successful formation of Cu_2O nanoparticles **(Badawy et al., 2015; Regmi et al., 2019; Lâm et al., 2022)**. The highest peak at about 36.66° corresponds to (111) plane, showing a dominant crystallographic orientation. This is a typical behavior of Cu_2O nanostructures, as preferential growth at nanoscale is common **(Kerour et al., 2018; Alsoltani & Harbbi, 2023)**. Using the most intense peak corresponding to the (111) plane of Cu_2O at $2\theta = 36.66°$, and a FWHM of 1.01°, the average crystallite size was calculated to be approximately 8.3 nm **(Monshi et al., 2012; Fatimah et al., 2021)**. This confirms that the synthesized Cu_2O consists of well-crystallized nanoparticles in the nanometer range **(Uddin et al., 2024; Lâm et al., 2022).(Figure 11)**

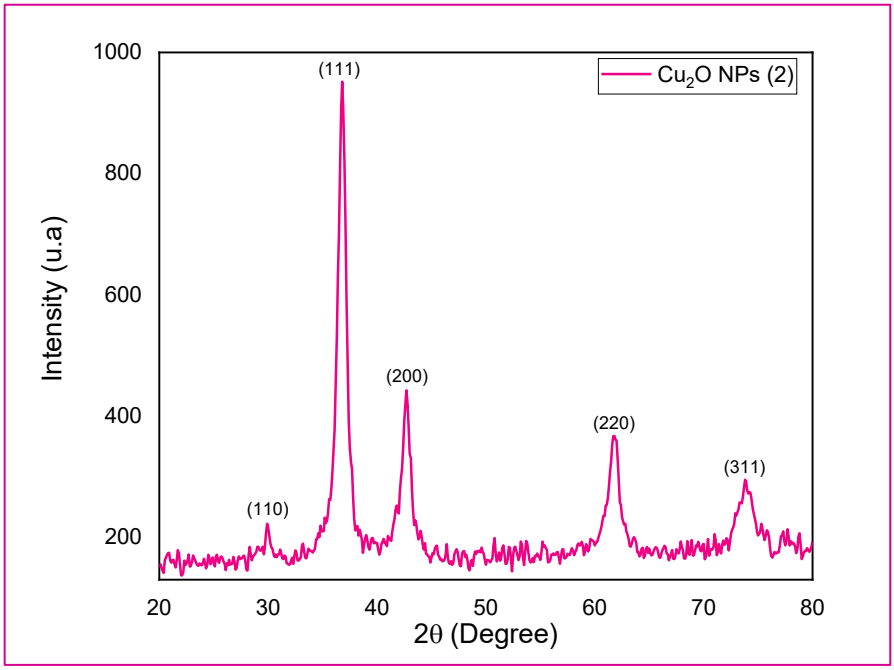


Figure 11: XRD pattern of CuONPs-BCC

1.1.4. SEM studies

The scanning electron microscopy (SEM) present the shape and surface characteristics of CuONPs (A) and CuONPs-BCC (B). As shown in Figure 12, The CuONPs exhibited relatively flat particles. In contrast, the CuONPs-BCC sample showed a more complex structure, with irregular particles forming rough surfaces and clustered shapes.

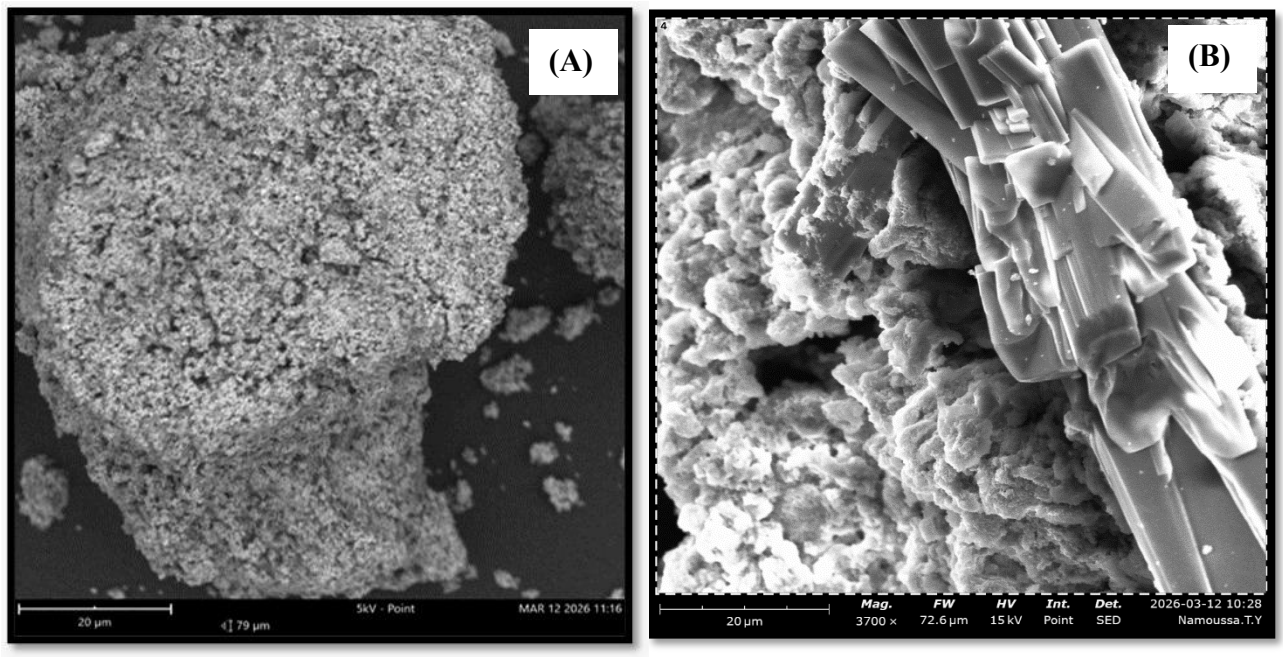


Figure 12: SEM micrographs *of* CuONPs (A) *and* CuONPs-BCC (B)

1.1.5. EDX studies

Energy dispersive X-ray (EDX) analysis of CuONPs (A) showed the presence of carbon, copper, oxygen, and nitrogen, indicating that copper nanoparticles were successfully formed and associated with organic components. On the other hand, the CuONPs-BCC sample (B) revealed a wider range of elements, including oxygen, copper, carbon, nitrogen, sulfur, calcium, sodium, and traces of tellurium. This confirms the presence of additional components in the BCC sample and suggests a more complex composition, likely due to the biological material used during synthesis.

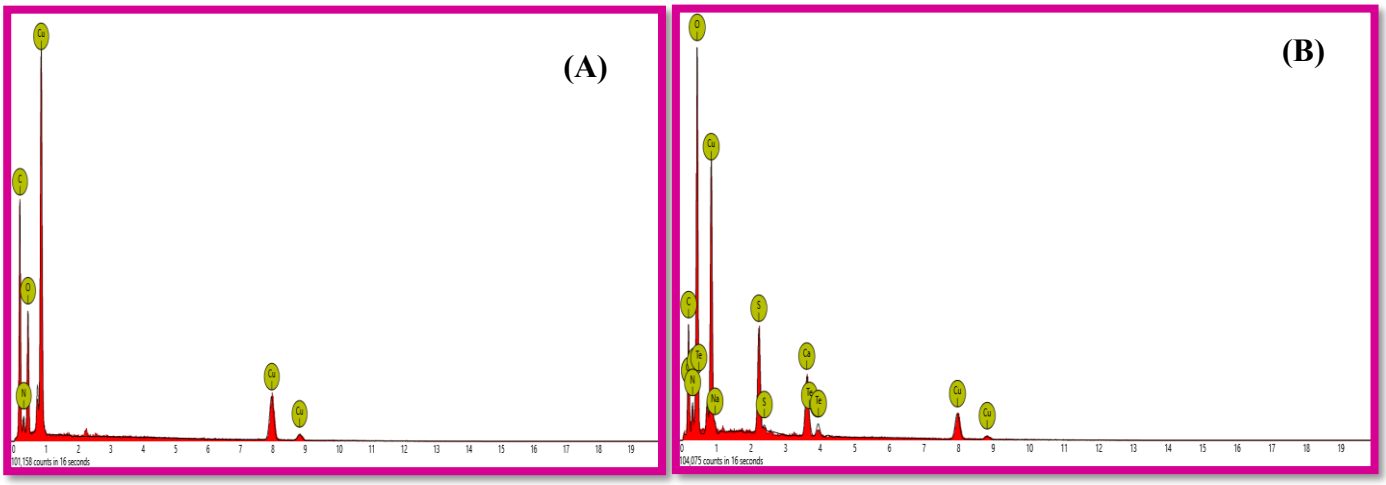


Figure 13: EDX analyses of the biosynthesized CuONPs (A) *and* CuONPs-BCC (B)

Table 1: The elemental composition analyses of CuONPs (A) from the EDX plot of the SEM images

Element Symbol	Element Name	Atomic Conc	Weight Conc.
C	Carbon	61.52	39.62
Cu	Copper	11.13	37.93
O	Oxygen	17.89	15.35
N	Nitrogen	9.45	7.10

Table 2: The elemental composition analyses CuONPs-BCC (B) from the EDX plot of the SEM images

Element Symbol	Element Name	Atomic Conc	Weight Conc.
O	Oxygen	45.33	37.30
Cu	Copper	6.29	20.55
C	Carbon	29.57	18.27
N	Nitrogen	11.77	8.48
S	Sulfur	3.20	5.27
Ca	Calcium	2.44	5.03
Te	Tellurium	0.64	4.20
Na	Sodium	0.77	0.91

1.2. Antioxidant Activity (Ferric Reducing Ability "FRAP")

Figure 14 represented the antioxidant activity based on the variation in IC50 values among the tested samples. Ascorbic acid recorded the lowest IC50 value (51 ug/ml), followed by CuONPs-BCC (66 ug/ml), whereas CuONPs exhibited the highest IC50 value (80 ug/ml) among all samples, indicating that CuONPs-BCC display better antioxidant activity compared to CuONPs.

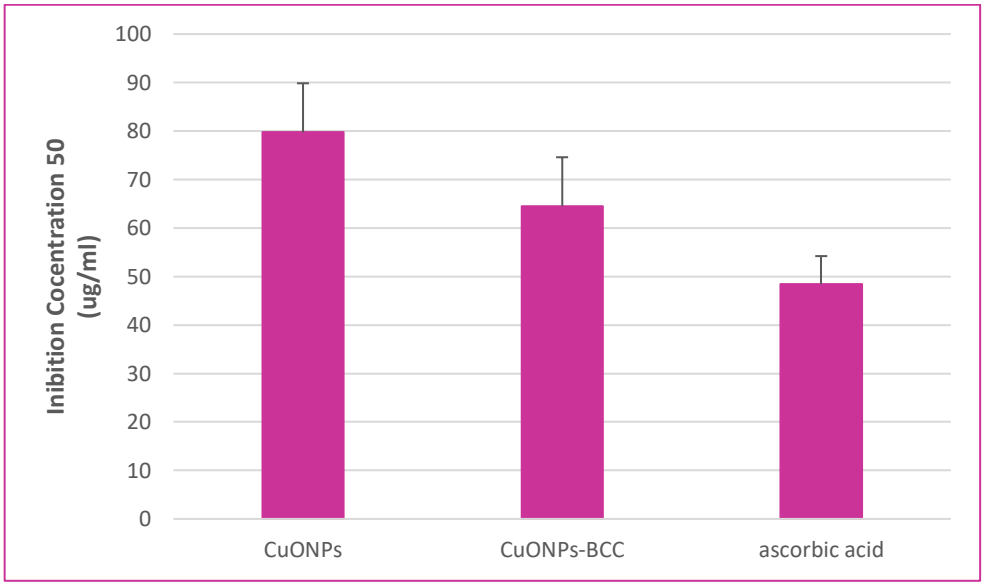


Figure 14: IC50 levels of antioxidant activity of CuONPs, CuONPs-BCC and ascorbic Acid.

1.3. Anti-inflammatory Activities

- **Anti-hemolytic activity**

In Figure 15, the results showed that CuONPs exhibit the strongest anti-hemolytic activity with the C50 value about 7 ug/ml, indicating. In contrast, CuONPs-BCC de the highest IC50 (14 ug/ml), while diclofenac demonstrated a moderate activity (10 ug/ml).

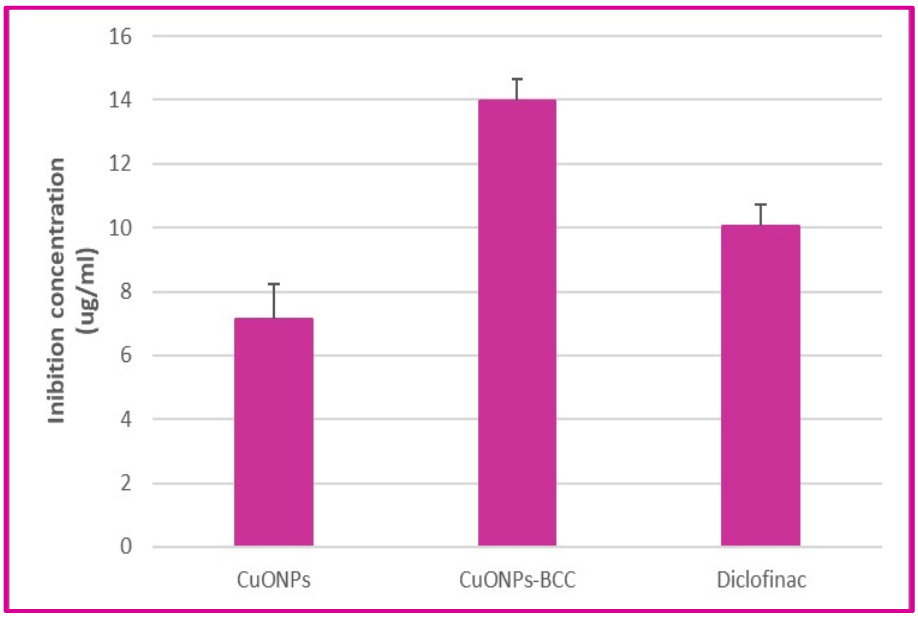


Figure 15: IC50 levels of anti-hemolytic activity of CuONPs-BCC , CuONPs and diclofinac.

❖ **Inhibition of Protein Denaturation Ability**

The results in Figure 16 showed that CuONPs-BCC have the lowest IC50 value (~70 µg/mL), indicating the highest effectiveness in inhibiting protein denaturation compared to the standard. However, CuONPs exhibit the lowest activity, while diclofenac showed intermediate effectiveness between them.

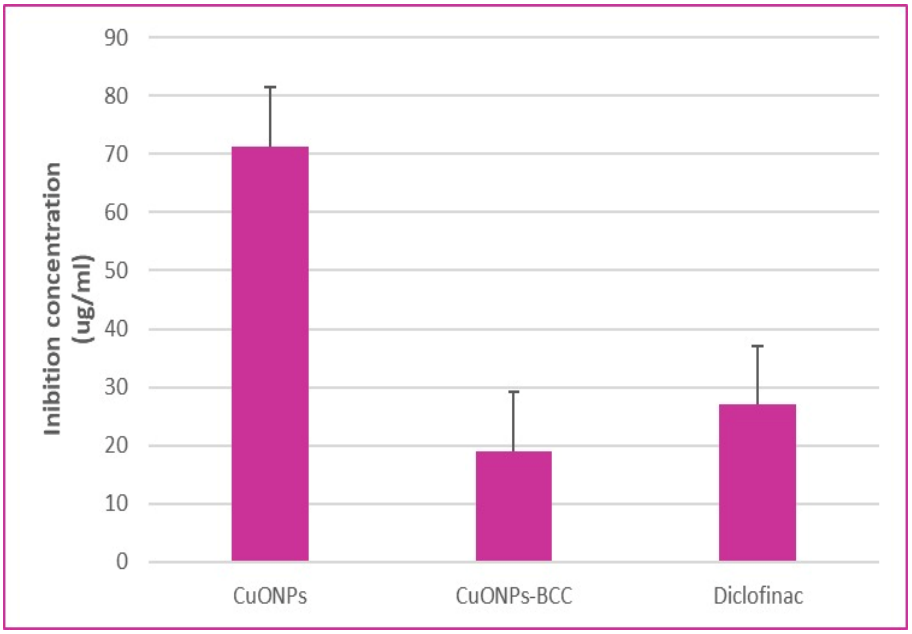


Figure 16: IC50 levels of inhibition of protein denaturation ability of CuONPs, CuONPs-BCC and diclofenac.

2. In Vivo study

2.1. In vivo Acute Toxicity Evaluation of CuONPs-BCC

The acute toxicity test using different doses of CuONPs-BCC (2.5 mg/kg and 5 mg/kg) did not demonstrate any changes in the behavioral and physiological parameters of the rats. No abnormal symptoms were observed during the observation and surveillance period, including any changes in the eyes, diarrhea, movement, and sleep disturbances, throughout 14 consecutive days. All animals showed no signs of toxicity, and no mortality was recorded during the experimental period **(Table 3**).

Table 3: Itraperitoneally acute toxicity of CuONPs-BCC on mortality and physiological parametres of albino Wistar rats .

Parameter	Doses	0 hours	3 hours	6 hours	24 hours	7 days	14 days
Eyes	Control	N	N	N	N	N	N
	2.5mg/kg	N	N	N	N	N	N
	5mg/kg	N	N	N	N	N	N
Sleep	Control	N	N	N	N	N	N
	2.5mg/kg	N	N	N	N	N	N
	5mg/kg	N	N	N	N	N	N
Movement	Control	N	N	N	N	N	N
	2.5mg/kg	N	N	N	N	N	N
	5mg/kg	N	N	N	N	N	N
Diarrhea	Control	N	N	N	N	N	N
	2.5mg/kg	N	N	N	N	N	N
	5mg/kg	N	N	N	N	N	N
Mortality	Control	Non	Non	Non	Non	Non	Non
	2.5mg/kg	Non	Non	Non	Non	Non	Non
	5mg/kg	Non	Non	Non	Non	Non	Non

N: Normal

2.2.Growth parameters

Relative organ weights is summarized in Table of the four groups (Control, Cisplatine, CuONPs-BCC, and BCC), the results in Table VI.4 presented that there were significant increases of relative organ weights; liver ($p<0.001$), heart ($p<0.001$), spleen ($p<0.01$) and kidneys ($p<0.05$) in the cisplatine group compared to the control group. While we noticed significant decreases of relative ogans weight in the CuONPs-BCC group ($p<0.05$)compared to the cisplatine group, with values approaching those of the control group. Similarly, a slight improvement was observed in the BCC group, particularly in the liver and spleen, although the effect was less pronounced compared to the CuONPs-BCC group.

Table 4 illustrates that in body wheight, compared to the control, there was a significant decrease in body weight ($p<0.01$) within the cisplatin group. However, in the CuONPs-BCC rats, body weight showed a very high significant increase ($p<0.001$) compared to cisplatin rats, with values approaching those of the control group. Likewise, the BCC group exhibited a marked raise ($p<0.01$) compared to the cisplatin group, indicating an improvement in body weight alterations induced by cisplatin toxicity.

Table 4: Growth parameters of the control and all experimental groups

Organ	Control (n=5)	Cisplatin (n=5)	CuONPs-BCC (n=5)	BCC (n=5)
Relative Liver Weight (%)	2.714±0.0372	3.0317±0.0358***	2.8653±0.339**b	2.537±0.105b
Relative Heart Weight (%)	0.29268±0.00976	0.324±0.00107***	0.304±0.00365**c	0.316±0.0122
Relative Spleen Weight (%)	0.2407±0.0133	0.2660±0.00132***	0.24350±0.00888a	0.24425±0.00699a
Relative Kidneys Weight (%)	0.5780± 0.0165	0.60900±0.00936*	0.5703±0.0142a	0.5680±0.0108a
Body Weight (g)	177.67±2.74	170.33±1.84**	184.67±1.28**c	183.67±2.49b

Values are mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001: significantly different from **control group**. **a** p<0.05, **b** p<0.01, **c** p<0.001: significantly different from **cisplatin group**.

2.3. Hematological parameters

The results presented in Table 5 indicate that the cisplatin group showed a significant decrease in WBC levels ($p < 0.01$) and lymphocyte counts ($p < 0.001$) compared with the control group. However, compared with the cisplatin group, both the CuONPs-BCC and BCC groups showed a significant increase in WBC levels ($p < 0.05$), restoring values closer to those of the control group. Regarding lymphocytes, a significant increase was observed in the BCC-treated rats ($p < 0.05$) compared with the cisplatin group.

Table 5: Leukocyte line in blood of the control and all experimental groups

Parameters	Control (n=5)	Cisplatine (n=5)	CuONPs-BCC (n=5)	BCC (n=5)
WBC (10^3/µl)	7.7±0.0365	6.233±0.327**	7.950±0.553a	7.675±0.502a
LYMPH (10^3/µl)	73.400±0.794	64.767±0.415***	64.1±0.668***	69.93±1.58a

Values are mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001: significantly different from **control group**.
a p<0.05, **b** p<0.01, **c** p<0.001: significantly different from **cisplatin group**.

Table 6 illustrates that, compared to the control, there was a significant decrease in RBC (p<0.05), HGB (p<0.01), and HCT (p<0.001) levels within the cisplatin group, accompanied by a high significant

increase in PLT ($p<0.001$) and MCV ($p<0.001$) levels. However, in the CuONPs-BCC rats, the levels of RBC, HCT and MCV showed a very high significant increase ($p<0.001$) and significant rise for HGB levels ($p<0.05$) , while a very high significant decrease was observed in PLT ($p<0.05$) levels compared to cisplatin rats. For the BCC group, a significant decrease was recorded in HGB ($p<0.001$) MCV ($p<0.001$) and PLT ($p<0.05$) compared to the control group.

Table 6: Erythrocyte and platelet line in blood of the control and all experimental groups

Parameters	**Control (n=5)**	**Cisplatine (n=5)**	**CuONPs-BCC (n=5)**	**BCC (n=5)**
RBC (10^6/µl)	6.637±0.107	6.386±0.113*	6.91±0.0273^{***c}	6.428±0.153
HGB (g/dl)	14.033±0.192	13.520±0.139**	13.867±0.166^{a}	13.2±0.0667^{***c}
HCT (%)	36.6±0.728	34.960±0.208***	37.700±0.167^{***c}	35.1±0.664
MCV (fl)	55.167±0.148	57.7±0.966***	54.167±0.223^{**c}	53.8±0.650^{c}
PLT (10^3/µl)	860.7±52.3	1084.3±17.2***	920±17.8^{*c}	950.3±51.7

Values are mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$: significantly different from **control group**.

a $p<0.05$, **b** $p<0.01$, **c** $p<0.001$: significantly different from **cisplatin group**.

2.4. Biochemical parameters

2.4.1.Blood glucose

Results in Figure 17 presented there was a significant increase in blood glucose ($p<0.001$) levels within the cisplatin group compared to the control,. However, in the CuONPs-BCC rats, blood sugar levels showed a significant decrease ($p<0.05$) compared to cisplatin rats, with values approaching those of the control group. For the BCC group, a moderate decrease in blood glucose levels was also observed compared to the cisplatin group.

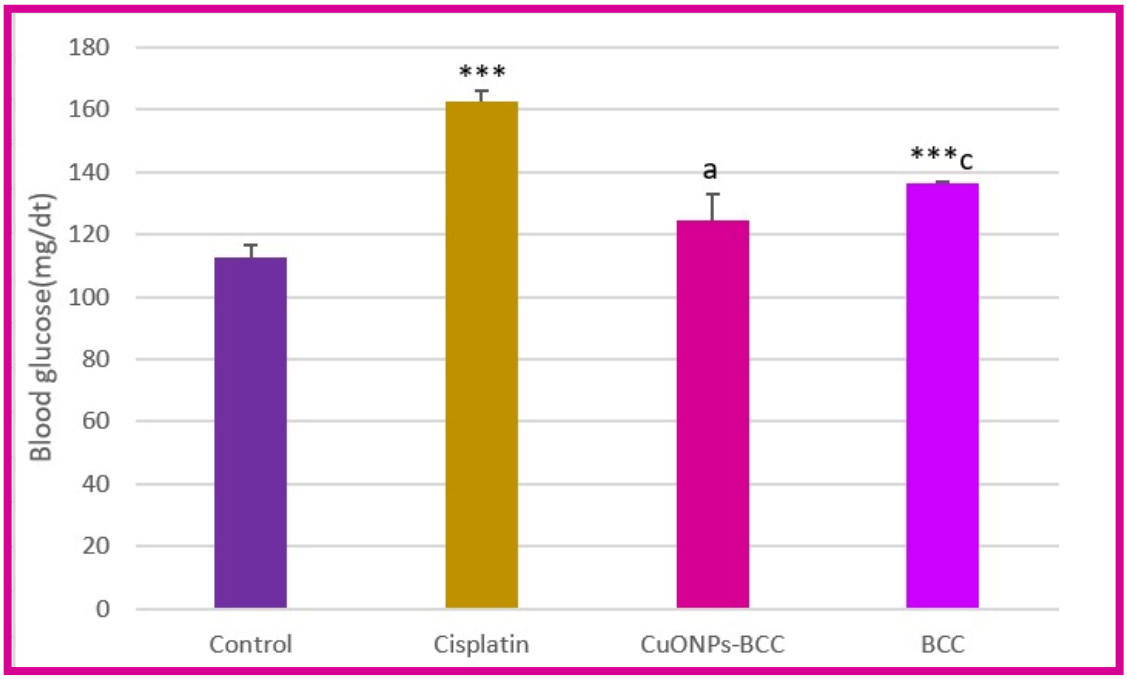


Figure 17: Blood glucose levels in control and experimental groups

2.4.2.CRP

Concerning CRP results in Figure 18 , the findings revealed that CRP levels in cisplatin group were very high significantly increased ($p<0.001$) compared to the control. However, CuONPs-BCC and BCC rats, CRP levels showed a very high significant decrease ($p<0.001$) compared to cisplatin rats. In fact, the effect of BCC was less pronounced than that observed in the CuONPs-BCC group.

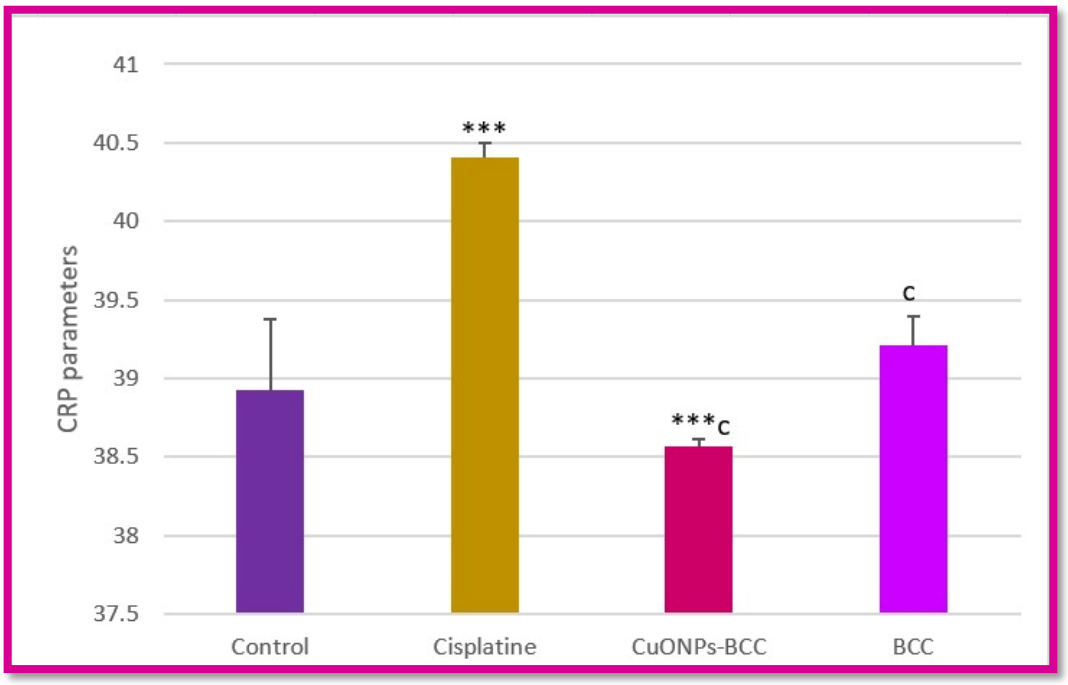


Figure 18: CRP Levels in control and experimental groups

2.4.3. IgG

The results show that the Cisplatin group had a slight increase in IgG levels compared with the Control group. The CuONPs-BCC treatment recorded the highest IgG value among all groups, with a clear significant difference in comparison to cisplatin and control group group. In contrast, the BCC group showed IgG levels that remained relatively close to those observed in the Cisplatin group (Figure 19). Overall, these findings indicate that CuONPs-BCC have a better positive effect on improving the immune response.

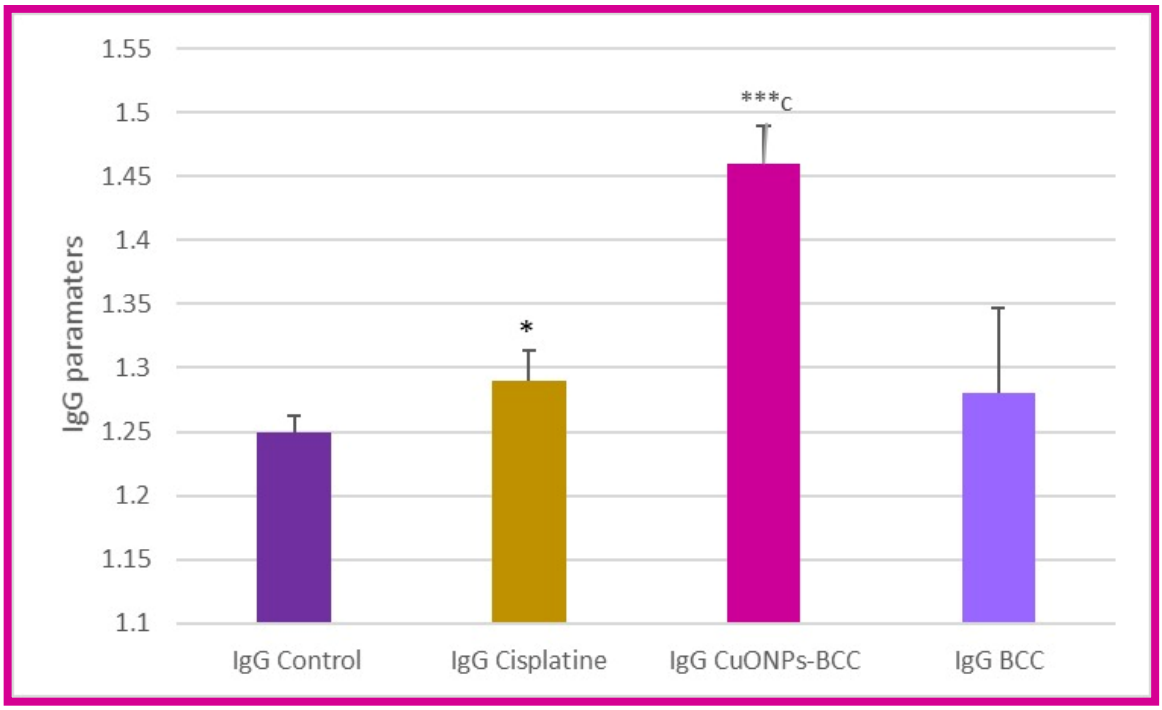


Figure 19: IgG Levels in control and experimental groups

2.5. Oxidative stress parameters

2.5.1.Malondialdehyde (MDA) level

Figure 20 demonstrated significant changes in MDA levels among all experimental groups compared with the control group. Cisplatin treatment increased MDA levels in all organs, with a very high significant increase observed in the liver ($p < 0.001$), a high significant increase in the heart and kidney ($p < 0.01$), and a significant increase in the spleen ($p < 0.05$) compared to control group. Treatment with CuONPs-BCC significantly decreased the MDA levels in all organs compared to the cisplatin group. These decreases were very high significant in the heart, spleen and liver ($p < 0.001$) and significant in the kidney ($p < 0.05$). BCC treatment also decreased MDA levels, mainly in the heart ($p < 0.001$), liver ($p < 0.001$), and spleen ($p < 0.05$), while the kidney showed smaller changes. In general, these results

suggest that both CuONPs-BCC and BCC help reduce MDA levels and mitigate the lipid peroxidation caused by cisplatin.

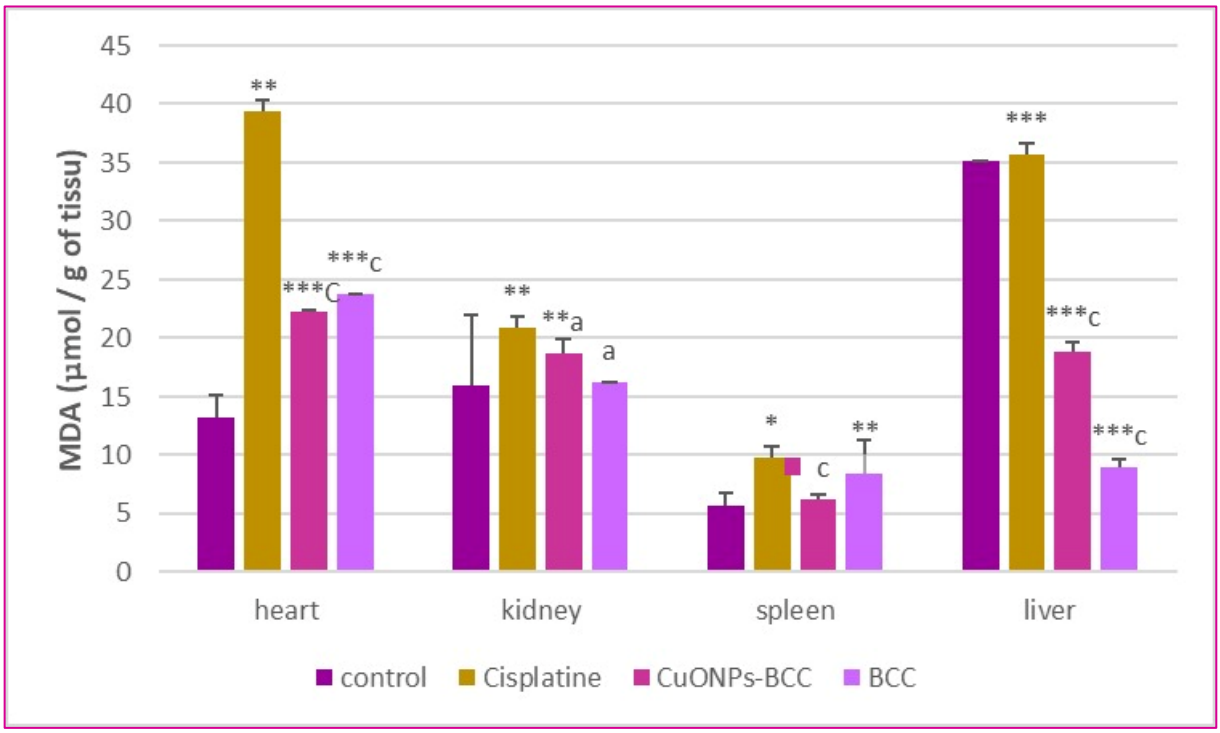


Figure 20: MDA levels in the control and all experimental groups

Values are mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001: significantly different from **control group**. **a** p<0.05, **b** p<0.01, **c** p<0.001: significantly different from **cisplatin group.**

2.5.2.Reduced glutathione (GSH) level

The results in Figure 21 showed changes in GSH levels in the different groups compared to the control group. In the cisplatin group, there was a very high significant decrease ($p < 0.001$) in GSH in heart, kidney, liver and spleen. Treatment with CuONPs-BCC significantly increased GSH levels in all organs compared to cisplatin group, particularly in the liver, which showed a highly significant increase ($p < 0.001$), with the exception of the spleen. For the BCC group, there was a significant increase in GSH levels in the heart ($p < 0.05$), spleen ($p < 0.01$) and kidney ($p < 0.01$), while the liver showed a significant decrease ($p < 0.001$) in comparison to cisplatin group. Overall, these results indicate that CuONPs-BCC improves GSH levels and reduces the oxidative damage caused by cisplatin in most organs.

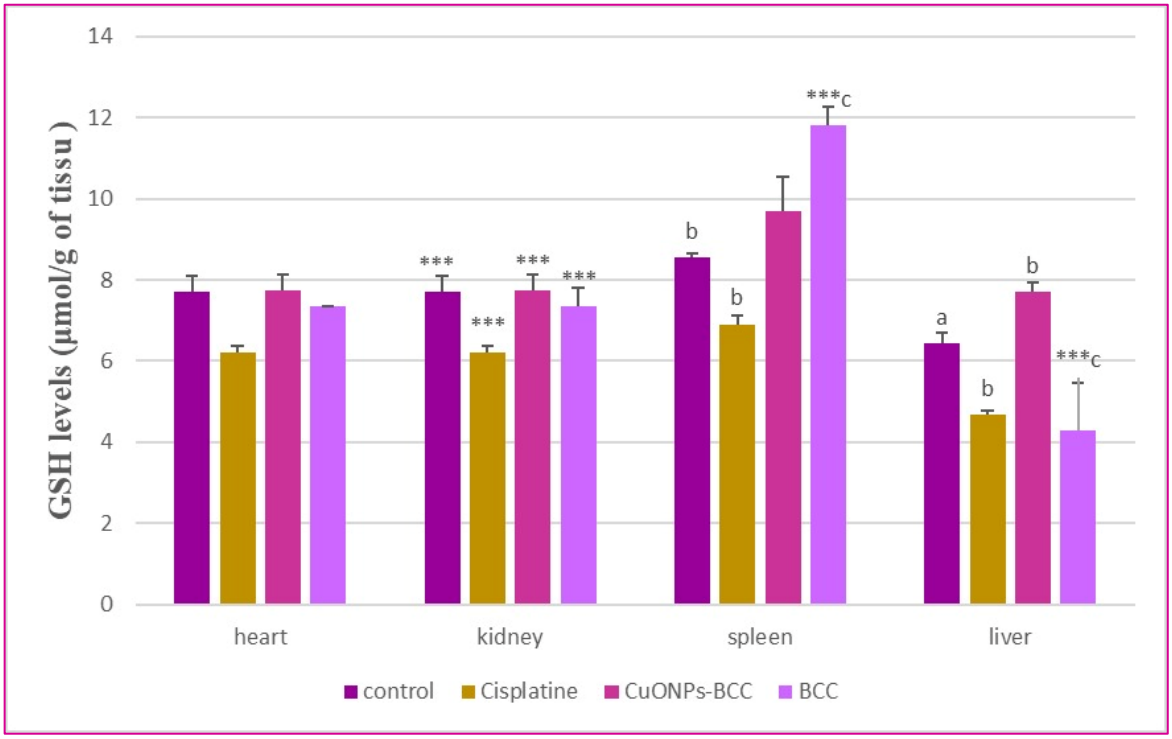


Figure 21:GSH levels in the control and all experimental groups

Values are mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001: significantly different from **control group**. **a** p<0.05, **b** p<0.01, **c** p<0.001 : significantly different from **cisplatin group**.

2.5.3.Superoxide dismutase (SOD) activity

Figure 22 presented the changes of SOD activities among the different treated groups compared to the control group. In the cisplatin-treated group, there was a significant decrease in SOD activities in all organs, especially in the heart and liver ($p < 0.001$), as well as in the spleen ($p < 0.05$). In the CuONPs-BCC group, SOD activities were increased compared to the cisplatin group in all organs. This increase was significant in the liver ($p < 0.001$), spleen ($p < 0.001$) and heart ($p < 0.05$), with values getting closer to the control group. For the BCC group, SOD activities also showed an increase, mainly in the liver ($p < 0.001$) and spleen ($p < 0.001$), while the heart and kidney showed smaller changes. Overall, these results suggest that both CuONPs-BCC and BCC help improve SOD levels and reduce the effect of oxidative stress caused by cisplatin.

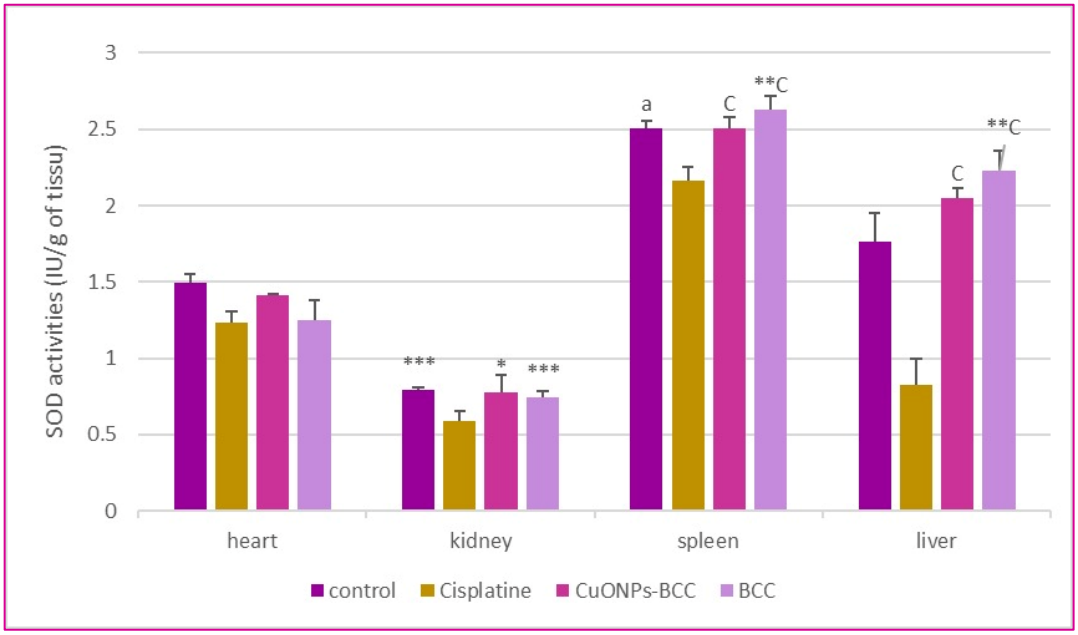


Figure 22: SOD activities in the control and all experimental groups

Values are mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001: significantly different from **control group**. **a** p<0.05, **b** p<0.01, **c** p<0.001: significantly different from **cisplatin group**.

2.6. Histopathological examination results

2.6.1.Liver

Liver sections from the control group displayed normal hepatic architecture, with hepatocytes arranged in well-defined hepatic cords. The cells appeared healthy with no signs of damage. In the cisplatin group, severe histopathological changes were observed. These included hepatocellular necrosis, inflammatory cell infiltration, and hemorrhage. The normal arrangement of hepatic cords was disrupted, indicating significant liver injury. The CuONPs-BCC treated group showed a marked improvement in liver structure. Hepatocytes appeared more regularly arranged, and necrotic areas were reduced. Inflammatory infiltration was also decreased. The BCC group showed partial improvement, with some restoration of hepatic architecture. However, mild necrosis, and limited inflammatory changes and hemorrhage were still observed, suggesting incomplete recovery. So, treatment by CuONPs-BCC showed the best amelioration compered to BCC group as presented in **figure 23**.

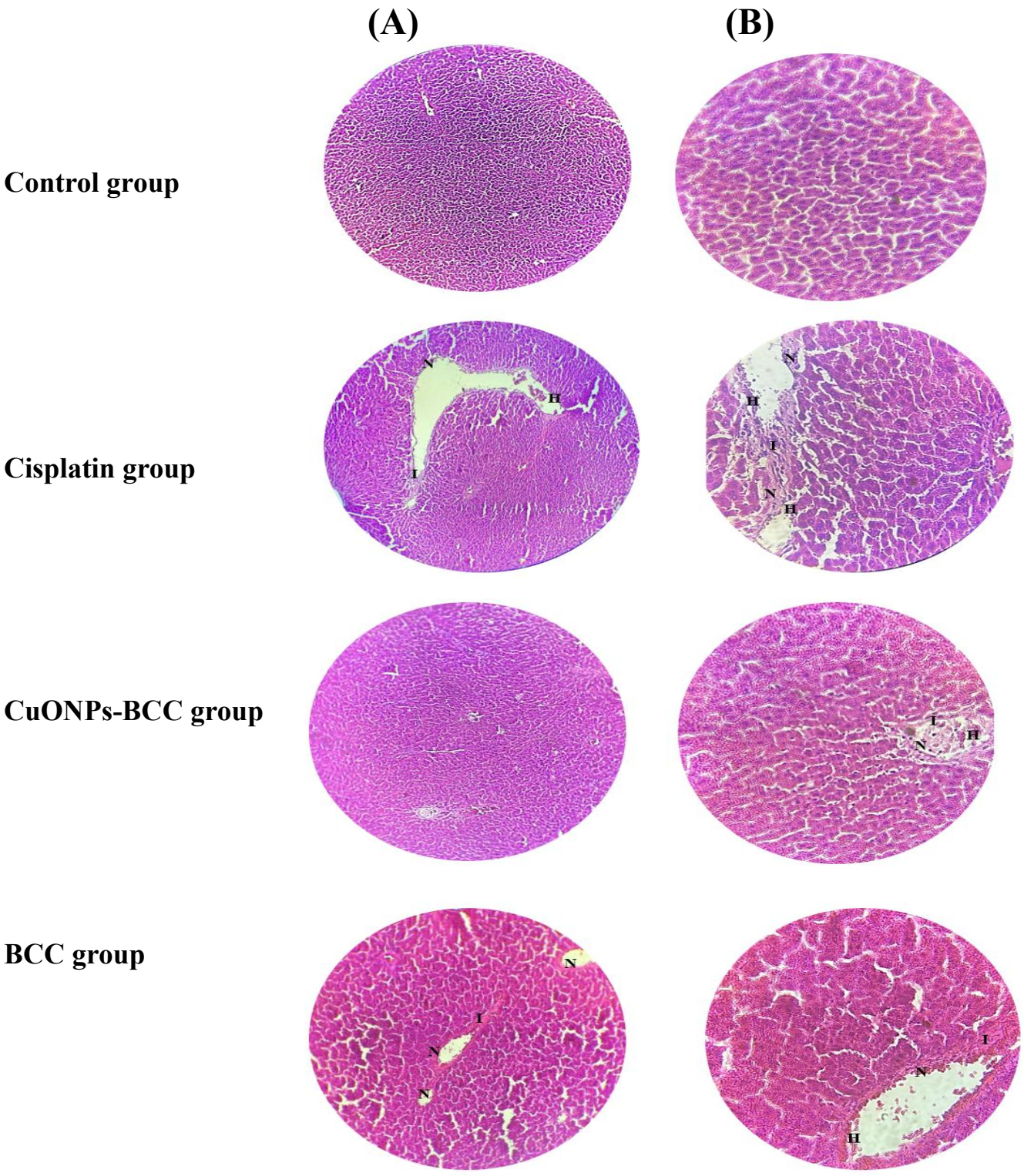
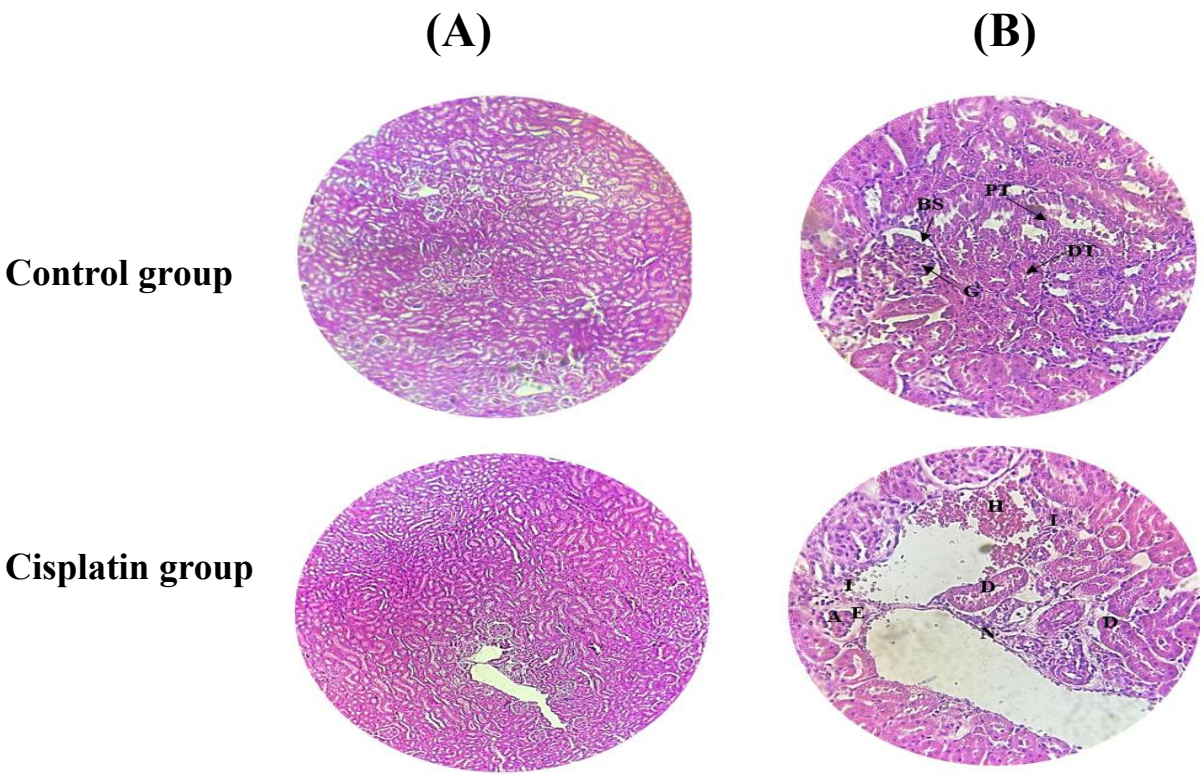


Figure 23: Photomicrographs of liver section of all experimental groups stained with hematoxylin and eosin **(A):** x10 and **(B)**: x40

H: hemmorage **I:** inflammatory **N:** necrosis

2.6.2.Kidneys

The control group showed a well-preserved renal architecture, with normal glomerulus, narrow Bowman's space, clearly defined proximal and distal convoluted tubules, and no signs of damage were observed. In the cisplatin group, marked structural alterations were evident. There was a clear dilation of Bowman's space, along with glomerular atrophy and distortion. The renal tubules showed dilation and epithelial damage, accompanied by areas of necrosis, hemorrhage, and significant infiltration of inflammatory cells, reflecting severe renal injury. In contrast, the CuONPs-BCC treated group showed a considerable improvement in kidney structure. Most glomerulus regained a near-normal appearance, Bowman's space was reduced compared to the cisplatin group, and the tubules appeared more organized with fewer signs of degeneration. Inflammatory infiltration and necrosis were also noticeably reduced. The BCC group showed partial recovery. Although some glomerulus and tubules appeared improved, there were still observable alterations such as mild dilation of Bowman's space, slight tubular damage, and limited areas of necrosis, indicating incomplete restoration. Globally, CuONPs-BCC presented a better amelioration than BCC in **Figure 24**.



CuONPs-BCC group

BCC group

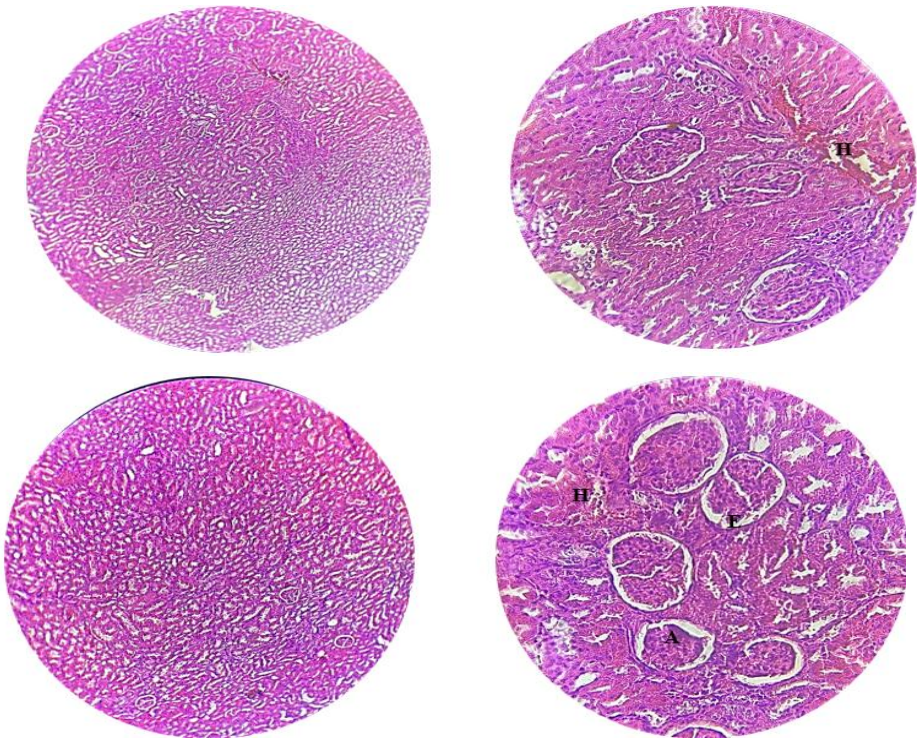


Figure 24: Photomicrographs of kidney section of all experimental groups stained with hematoxylin and eosin **(A):** x10 and **(B)**: x40

G: Glomerulus, **BS**: Bawman's space, **PT**: Proximal tubules, **DT**: Distal tubules
I: Inflammation, **H**: Hemorrhage, **N:** Necrosis, **E:** Bawman's space expansion, **D:** Renal tubules dilatation, **A:** Glomerulus atrophy

2.6.3. Heart

Figure 25 showed that control group exhibited normal cardiac histology, with well-organized myocardial fibers, intact cell structure, and no evidence of inflammation or degeneration. In the cisplatin group, the heart tissue showed pronounced pathological changes, including disorganization and degeneration of myocardial fibers and noticeable collagen accumulation. The CuONPs-BCC and BCC treated group demonstrated clear improvement, as the myocardial fibers appeared more regularly arranged, with reduced degeneration. Collagen deposition was also less evident compared to the cisplatin group.

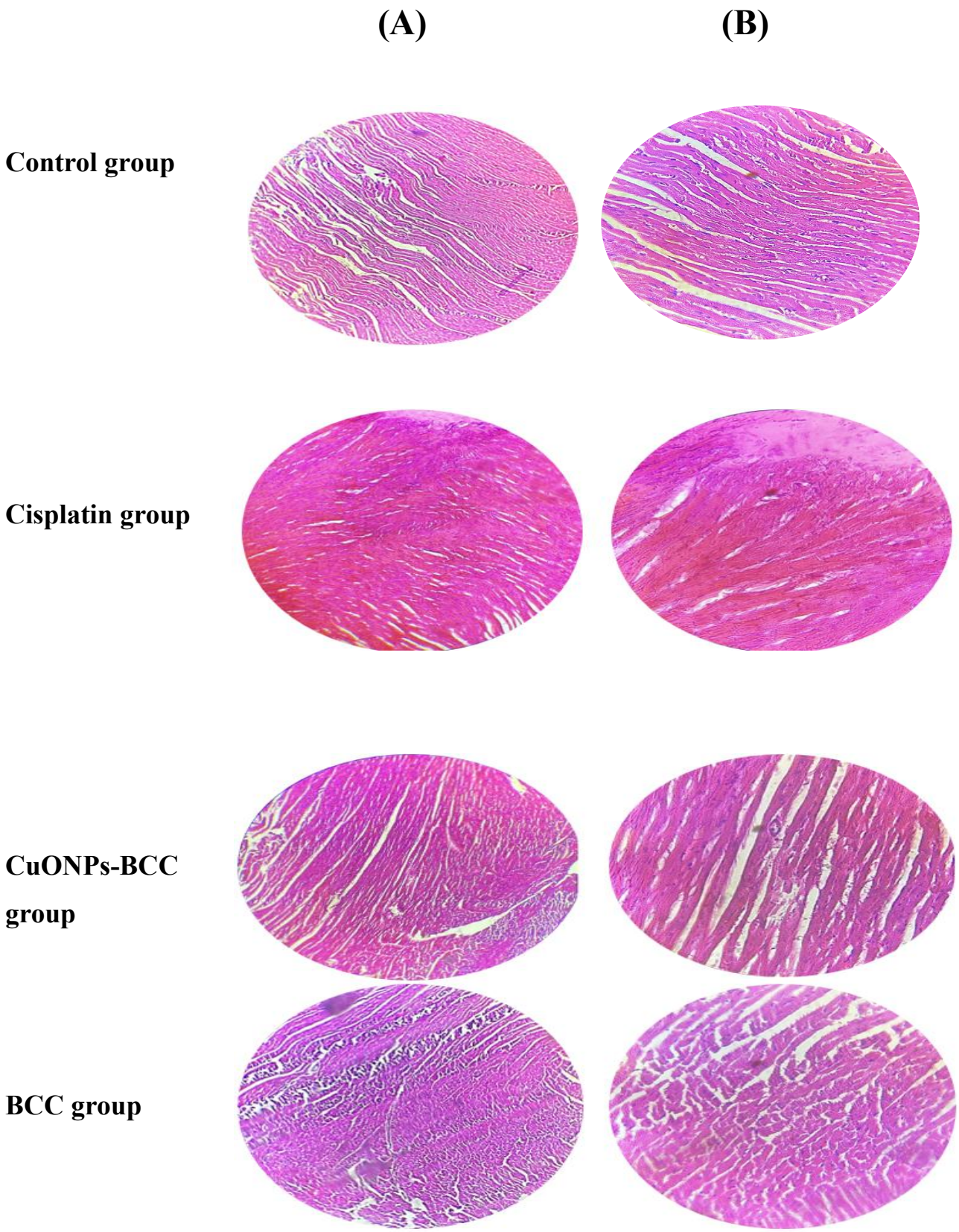
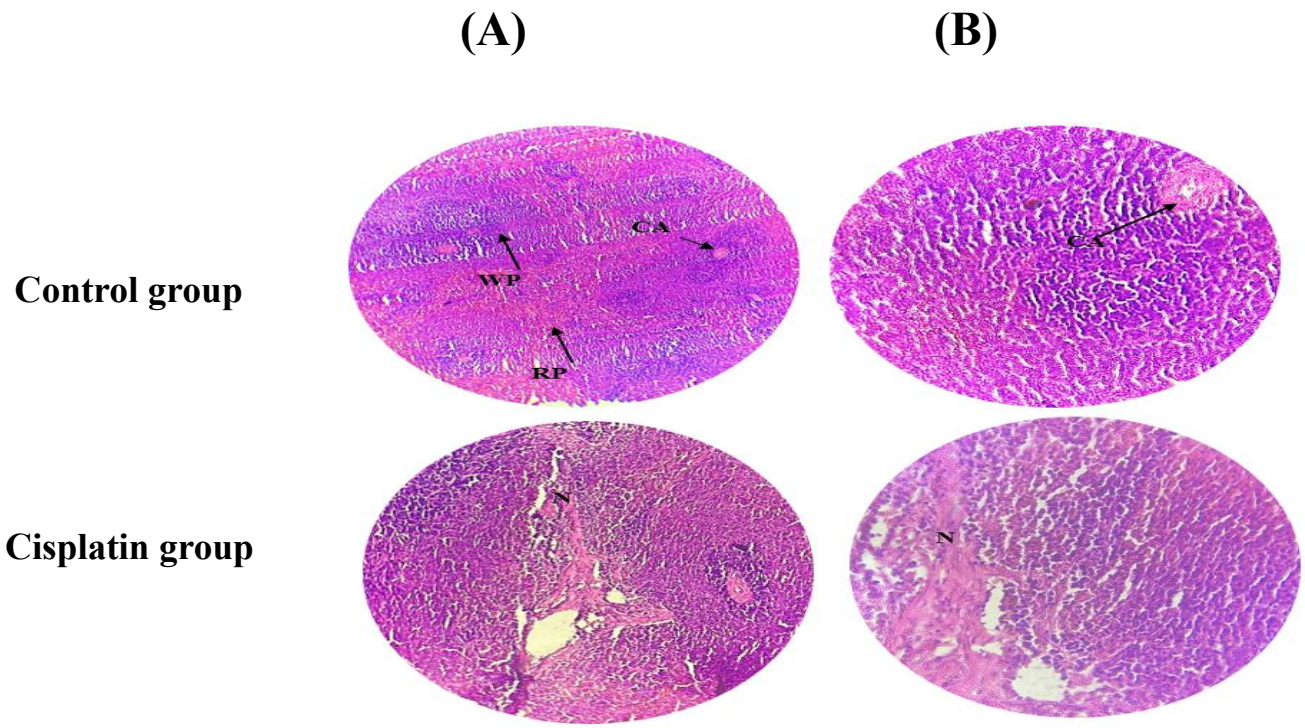


Figure 25: Photomicrographs of heart section of control and experimental groups stained with hematoxylin and eosin; **(A):** x10 and **(B)**: x40.

2.6.4.Spleen

Spleen sections of control group showed normal histological organization, with clearly distinguishable white pulp and red pulp regions, and no structural abnormalities. In the cisplatin group, the splenic architecture was disrupted. There were areas of necrosis and disorganization, particularly affecting the white pulp, along with a reduction in its normal structure. These changes indicate impaired splenic function. The CuONPs-BCC treated group showed improvement in splenic tissue, with partial restoration of the white pulp and better organization of the red pulp. The extent of necrosis was reduced compared to the cisplatin group. The treatment by BCC exhibited partial recovery, where some normal features were restored (Figure 26); these results translate efficacy of our therapeutic systems.



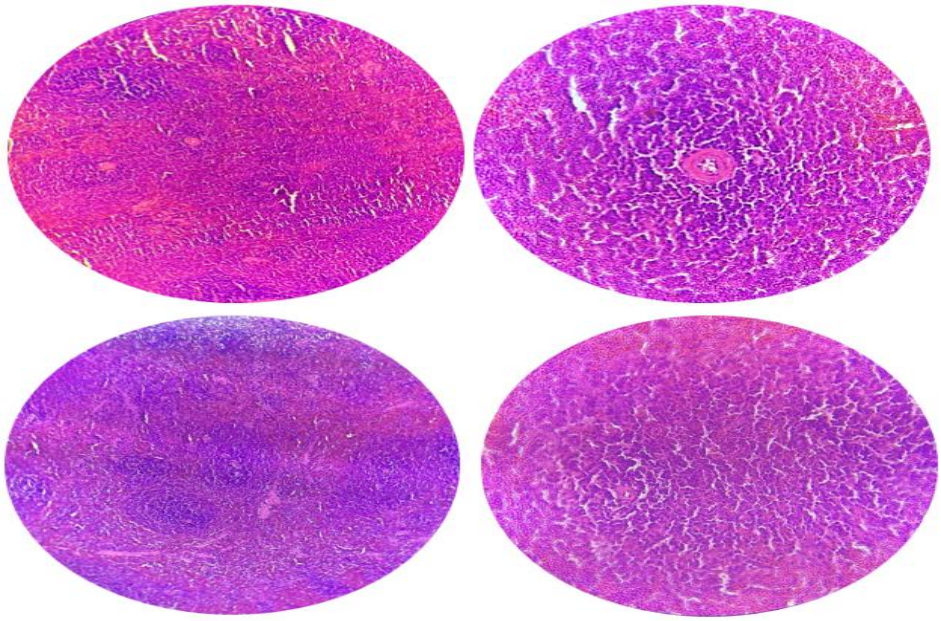


Figure 26: Photomicrographs of spleen section of control and experimental groups stained with hematoxylin and eosin; **(A):** x10 and **(B):** x40.

RP: Red pulp, **WP:** White pulp, **CA:** central arteriol, **N:** Necrosis

II. Discussion

Breast cancer is one of the most prevalent and life-threatening cancers worldwide, characterized by the uncontrolled proliferation of abnormal breast cells and their ability to evade immune defense mechanisms (Siegel et al., 2024). Conventional chemotherapy remains widely used in breast cancer treatment; however, it is frequently associated with severe side effects and immune suppression (Liu et al., 2023). Recently, nanotechnology-based approaches have attracted significant attention due to their ability to improve therapeutic targeting and enhance anticancer immune responses (Wang et al., 2024). In this study, single-cell nanocapsulation was investigated as an innovative strategy for strengthening the immune system against breast cancer using biocompatible nanomaterials (Zhang et al., 2023). Several in-vitro and in-vivo experiments were performed to evaluate the immunomodulatory and anticancer activities of the developed nanomaterials (Chen et al., 2022).

1. Characterization of CuONPs

The UV–Vis analysis of CuONPs in the present study showed an absorption peak around 240 nm, which indicates the formation of copper-containing nanostructures associated with HSA. Similar findings were reported by Amaliyah et al. (2020), who observed an absorption peak near 250 nm during the characterization of biosynthesized copper nanoparticles. The authors suggested that this absorption behavior was related to the successful formation and stabilization of copper nanoparticles. The slight difference in peak position between the present study and previous reports may be attributed to

differences in synthesis conditions, nanoparticle size, or the nature of the stabilizing biomolecules used during nanoparticle preparation.

The FTIR spectra of CuONPs and CuONPs-BCC recorded in the range between 400 and 4000 cm^{-1}(Amaliyah et al. 2020). FTIR analysis was used to identify the functional groups involved in the synthesis and stabilization of copper nanoparticles.(Mohammed et al. 2025). A clear absorption band appeared between 400 and 600 cm^{-1}, which confirms the formation of copper nanoparticles. (Mohammadi & Danafar 2023). Similar peaks related to copper nanoparticles were also reported in previous studies. (Mohammed et al. 2025). Tsilo et al. (2023) reported a peak at 680 cm^{-1} related to the interaction between biomolecules and copper nanoparticles (Tsilo et al. 2023). The broad bands observed around 3200–3400 cm^{-1} may correspond to O–H and N–H groups involved in nanoparticle stabilization. (Fatima et al. 2024). Similar observations were described by Tsilo et al (2023), who associated this region with hydroxyl and amine groups (Tsilo et al. 2023). Peaks detected near 1633 cm^{-1} may be related to amine or amide group vibrations, indicating the participation of protein functional groups in copper binding. (Mahanthappa et al. 2017). Bands observed around 1000–1100 cm^{-1} could be associated with C–O–C groups and other biological compounds acting as reducing or capping agents. (Amaliyah et al. 2020). The differences observed between the spectra of CuONPs and CuONPs-BCC suggest changes in their chemical composition after BCC incorporation (Mohammadi & Danafar 2023). Changes in peak intensity and position may indicate stronger interactions between copper nanoparticles and surrounding biomolecules. (Mahanthappa et al. 2017). Overall, the FTIR results confirm the successful synthesis of copper nanoparticles and highlight the role of biological molecules in nanoparticle stabilization (Fatima et al. 2024).

The XRD results confirmed the successful synthesis of crystalline Cu_2O nanoparticles with a cubic cuprite structure, as all diffraction peaks matched the standard JCPDS card No. 05-0667 (Li et al., 2023). The detected peaks at 2θ values of 30.32°, 36.66°, 42.76°, 61.81°, and 73.63° corresponded to the characteristic planes of Cu_2O, in agreement with previous studies on biologically synthesized Cu_2O nanoparticles (Ramesh et al., 2022).

The intense diffraction peak at 36.66° related to the (111) plane indicated preferential crystal growth along this direction, which is commonly observed in Cu_2O nanostructures (Zhang et al., 2021).Similar findings were reported by Wang et al. (2022), who showed that dominant exposure of the (111) facet enhances the surface activity and photocatalytic properties of Cu_2O nanoparticles.The sharp diffraction peaks also indicated high crystallinity and structural stability of the synthesized nanoparticles .(Chen et al., 2023).

The calculated crystallite size was approximately 8.3 nm, confirming the nanocrystalline nature of CuONPs -BCC .(Singh et al., 2023).This result is comparable to that reported by Abdel-Halim et al. (2024), who obtained Cu_2O nanoparticles with sizes ranging between 6–10 nm. The small crystallite size may improve the catalytic, adsorption, and biological properties of the nanoparticles due to the increased surface-to-volume ratio. (Rahman et al., 2023).Furthermore, the absence of impurity peaks related to CuO or metallic copper confirmed the high purity and phase stability of the synthesized nanoparticles (Mohamed et al., 2024).

SEM analysis was used to examine the morphology and surface characteristics of CuONPs and CuONPs-BCC (Aziz et al., 2025). The CuONPs-BCC sample showed relatively flat and more uniform particles, while CuONPs exhibited irregular and clustered structures with rough surfaces. Similar aggregation behavior has been reported previously for copper-based nanomaterials (Gontrani et al., 2023). These differences may be related to the presence of biological compounds that affected nanoparticle growth and surface organization during synthesis (Keabadile et al., 2020).

EDX analysis confirmed the elemental composition of both CuONPs and CuONPs-BCC samples (Aziz et al., 2025). The CuONPs spectrum showed the presence of carbon, copper, oxygen, and nitrogen, indicating the successful formation of copper nanoparticles associated with organic compounds (Sagadevan & Koteeswari, 2015). In contrast, CuONPs-BCC displayed additional elements such as sulfur, calcium, sodium, and traces of tellurium, suggesting the incorporation of biological materials and a more complex composition (Okpara et al., 2021). These findings indicate that BCC contributed to changes in the surface chemistry and elemental profile of the nanoparticles.

2. Discussion of FRAP assay

The results of the FRAP assay showed that CuONPs-BCC exhibited a significantly higher antioxidant capacity compared to CuONPs alone, indicating an enhancement in reducing power due to bioconjugation. This increased FRAP activity reflects a stronger ability of the bioconjugated nanoparticles to neutralize reactive oxygen species (ROS), which are the main contributors to oxidative stress.

The FRAP assay is based on the reduction of Fe^{3+} to Fe^{2+}, which reflects the electron-donating ability of antioxidant compounds and their capacity to neutralize free radicals (Apak, R., et al. 2016).

The FRAP assay results indicated that both CuONPs and CuONPs-BCC exhibited measurable antioxidant activity; however, the bioconjugated formulation (CuONPs-BCC) showed a markedly higher ferric reducing power compared to nanoparticles alone.

This suggests that while copper nanoparticles inherently possess redox activity due to their ability to participate in electron transfer reactions, their antioxidant efficiency is significantly enhanced after conjugation with BCC. The improved FRAP values in the CuONPs-BCC system can be attributed to a synergistic effect, where bioactive compounds present in BCC—such as phenolic and flavonoid molecules—act as additional electron donors, thereby increasing the overall reducing capacity. In contrast, CuONPs alone rely mainly on the intrinsic redox cycling of copper ions (Cu^{+}/Cu^{2+}), which provides moderate antioxidant activity but is less effective in neutralizing reactive oxygen species compared to the conjugated system. These findings are consistent with recent studies showing that functionalization of metal nanoparticles with bioactive compounds significantly enhances their antioxidant properties and redox stability (Ijaz et al., 2022; Wang et al., 2023).

Overall, the FRAP assay confirms that the incorporation of BCC not only improves the antioxidant potential of copper nanoparticles but also strengthens their ability to combat oxidative stress through enhanced electron-donating mechanisms.

3. Anti-inflammatory Activities

3.1. Anti-hemolytic activity

The results demonstrate that CuONPs exhibited the strongest anti-hemolytic activity, as evidenced by the lowest IC50 value (~7 µg/mL), indicating a high protective effect against erythrocyte membrane damage (Smith et al., 2021).

This finding is consistent with previous studies reporting that nanoparticles conjugated with human serum albumin enhance membrane stability and reduce hemolysis due to their biocompatibility and surface functionalization (Zhang et al., 2022).

In contrast, CuONPs-BCC showed the highest IC50 value (~14 µg/mL), suggesting a lower anti-hemolytic potential, which may be attributed to structural modifications affecting nanoparticle–membrane interactions (Kumar et al., 2023).

Diclofenac demonstrated moderate activity (~10 µg/mL), which aligns with its known anti-inflammatory properties but relatively limited membrane-protective effects compared to nanomaterials (Ahmed et al., 2021).

The anti-hemolytic effect observed can be explained by the ability of nanoparticles to inhibit oxidative stress-induced lipid peroxidation in erythrocyte membranes, thereby preventing hemolysis (Li et al., 2022).

Overall, these results suggest that CuONPs possess superior protective activity against hemolysis, making them promising candidates for biomedical applications where membrane stabilization and antioxidant defense are required (Wang et al., 2023).

3.2. Inhibition of Protein Denaturation Ability

The results obtained in Figure X demonstrated that CuONPs-BCC exhibited the highest anti-inflammatory activity, as indicated by the lowest IC_{50} value (~70 µg/mL) in the protein denaturation assay. This suggests that the synthesized nanocomposite has a strong ability to stabilize proteins and protect them from denaturation under stress conditions. Protein denaturation is closely related to inflammatory reactions, and inhibition of this process is considered an important indicator of anti-inflammatory potential. Similar findings were reported by Saeedi et al. (2025), who observed that copper nanoparticles exhibited significant anti-inflammatory activity due to their ability to reduce protein damage and oxidative stress.

The superior activity of CuONPs-BCC may be explained by the synergistic effect between copper nanoparticles and HSA functionalization, which enhances nanoparticle stability and improves interaction with biomolecules. In agreement with our findings, Vodyashkin et al. (2024) reported that functionalized copper nanoparticles demonstrated enhanced biological and anti-inflammatory activities because of their high surface reactivity and improved biocompatibility. Likewise, Yaqoob et al. (2023) found that engineered metal nanoparticles possess stronger anti-inflammatory properties than non-functionalized forms due to better cellular interaction and protein stabilization.

In contrast, CuONPs showed lower inhibitory activity, which may be due to weaker surface modification and possible nanoparticle aggregation, reducing the effective interaction with proteins. Similar observations were described by Yaqoob et al. (2023), who reported that insufficient nanoparticle functionalization decreases biological performance and limits anti-inflammatory efficiency.

also, diclofenac exhibited moderate activity, which may be related to its mechanism as a non-steroidal anti-inflammatory drug (NSAID). Diclofenac acts mainly through inhibition of cyclooxygenase enzymes (COX-1 and COX-2), thereby reducing prostaglandin synthesis rather than directly stabilizing proteins. Comparable results were reported by Srinivasan et al. (2022), who demonstrated that diclofenac shows effective anti-inflammatory activity but lower protein stabilization ability compared with some nanoparticle-based systems.

Overall, the present findings indicate that CuONPs-BCC possess enhanced anti-inflammatory potential compared to diclofenac and CuONPs, highlighting the importance of nanoparticle functionalization in improving biological activity and protein stabilization mechanisms.

4. Growth parameter

The significant increase in the relative weights of the liver, kidneys, heart, and spleen observed in the cisplatin-treated group may be attributed to oxidative stress, inflammation, edema, and cellular degeneration induced by cisplatin toxicity (Tang et al., 2023). Cisplatin is known to accumulate in highly perfused organs and stimulate excessive production of reactive oxygen species (ROS), leading to mitochondrial dysfunction and tissue injury (Tang et al., 2023).

The increase in relative liver weight may be explained by hepatocellular degeneration, inflammatory infiltration, and lipid peroxidation caused by cisplatin exposure (Elshopakey et al., 2022). Similar findings were reported by Elshopakey et al. (2022), who demonstrated that cisplatin induces severe hepatotoxicity associated with oxidative stress, apoptosis, and structural liver damage.

The significant elevation in kidney relative weight in the cisplatin group is mainly associated with nephrotoxicity because the kidneys are the primary site of cisplatin accumulation and excretion (Tang et al., 2023). Cisplatin-induced renal injury is characterized by tubular necrosis, oxidative stress, mitochondrial dysfunction, and inflammatory responses that result in renal edema and increased kidney mass (Lin et al., 2023). Likewise, Lin et al. (2023) demonstrated that cisplatin promotes renal tubular epithelial injury through oxidative stress and ferroptosis pathways.

The increase in relative heart weight may be related to cardiotoxic effects induced by oxidative stress and mitochondrial injury in cardiac tissues (El-Sawalhi et al., 2021). Cisplatin can stimulate inflammatory reactions and myocardial cell damage, leading to tissue swelling and cardiac structural abnormalities (El-Sawalhi et al., 2021).

The spleen also exhibited a significant increase in relative weight following cisplatin treatment, which may reflect immune dysfunction, splenic congestion, and inflammatory cell infiltration induced by oxidative stress (Zhao et al., 2023). Similar observations were described by Zhao et al. (2023), who reported that oxidative stress and inflammatory signaling contribute to pathological alterations in immune-related tissues.

In contrast, treatment with CuONPs-BCC significantly reduced the relative weights of these organs compared with the cisplatin-treated group, with values approaching those of the control group. This improvement may be attributed to the potent antioxidant and anti-inflammatory properties of the synthesized nanoparticles, which reduced ROS generation and attenuated tissue injury (Davoudi et al., 2022). Similarly, Davoudi et al. (2022) demonstrated that functionalized nanoparticles possess protective effects against chemotherapy-induced organ toxicity through suppression of oxidative stress and inflammatory pathways.

5. Hematological parameters

Our results demonstrated a significant decrease in leukocyte count in the cisplatin-treated group compared with the control group. The majority of immunological cells are thought to be leukocytes. These cells provide complete monitoring to many organs and tissues via the normal circulation of blood to lymph, from the inside of the arteries to the outside, and from the inside of the tissues to the blood, resulting in the body's defense against pathological elements (Ley, K.et al 2007) . This decrease may be attributed to the myelosuppressive effect of cisplatin, which reduces leukocyte production and weakens immune defense (Gao, Z.and al 2023). The present findings demonstrated that cisplatin administration caused significant alterations in leukocyte parameters, as shown by the marked decrease in WBC and LYMPH levels compared with the control group. These results indicate that cisplatin induced severe toxicity and immunosuppression, which may weaken the immune defense system and increase susceptibility to infections (Dasari et al 2014) .

The reduction in total white blood cell count (WBC) can be explained by the myelosuppressive effect of cisplatin, which inhibits bone marrow activity and decreases leukopoiesis (Elmorsy et al., 2024). In addition, cisplatin stimulates excessive production of reactive oxygen species (ROS), leading to oxidative stress, apoptosis of hematopoietic cells, and damage to leukocyte membranes (Oun et al., 2022). The significant decrease in lymphocyte count (LYMPH) further reflects the immunotoxic effect of cisplatin, since lymphocytes are highly sensitive to oxidative damage and DNA crosslinking caused by platinum-based drugs (Oun et al., 2018). Similar observations were reported by Dasari and Tchounwou (2014), who stated that cisplatin causes bone marrow suppression, leukopenia, and immune dysfunction through oxidative stress and cellular toxicity. Likewise, Almeer et al. (2022) reported that cisplatin-induced oxidative stress significantly reduced leukocyte and lymphocyte counts and caused inflammatory tissue damage. In addition, El-Shoura et al. (2024) demonstrated that antioxidant treatment alleviates cisplatin-induced immunotoxicity by reducing oxidative stress and restoring normal hematological parameters.

In contrast, treatment with bioconjugated CuONPs (CuONPs-BCC) and the BCC group showed improvement in WBC levels, maintaining values closer to the control group. This improvement may be explained by the antioxidant and cytoprotective properties of copper nanoparticles conjugated with human serum albumin, which help neutralize free radicals, reduce oxidative stress, preserve erythrocyte membrane integrity, and support normal hematopoietic activity (Ibrahim et al., 2023). Human serum albumin also enhances nanoparticle stability and biocompatibility, improving their therapeutic efficiency (Langer et al., 2003). Although the CuONPs-BCC group showed a decrease in lymphocyte count, the overall restoration of WBC levels indicates partial protection against cisplatin-induced

immunosuppression. The BCC group also showed a moderate improvement, but the effect was less pronounced than that observed in the CuONPs-BCC group, suggesting that the nanoparticle bioconjugation enhanced the protective efficacy. These findings suggest that CuONPs-BCC may serve as a promising protective strategy against cisplatin-induced leukocyte toxicity through antioxidant, anti-inflammatory, and immunomodulatory mechanisms.

The results of the present study demonstrated that cisplatin administration caused significant disturbances in erythrocyte and platelet parameters, as evidenced by the marked decrease in RBC, HGB, and HCT levels, along with a significant increase in MCV and platelet count (PLC) compared with the control group. These findings confirm the hematotoxic effect of Cisplatin and indicate the development of anemia and blood homeostasis imbalance The reduction in RBC, HGB, and HCT may be attributed to the myelosuppressive effect of cisplatin, which suppresses bone marrow activity and reduces erythropoiesis, leading to anemia and hematological toxicity (Miller et al., 2022). In addition, cisplatin induces excessive production of reactive oxygen species (ROS), leading to oxidative stress, lipid peroxidation, and erythrocyte membrane damage, which accelerate red blood cell destruction and shorten their lifespan (Işeri et al., 2021).These findings are consistent with the study of Abdel-Latif et al. (2022), who reported that cisplatin-induced toxicity is strongly associated with oxidative stress and cellular damage, resulting in significant hematological disturbances. The significant increase in MCV observed in the cisplatin-treated group may reflect abnormal erythrocyte maturation and macrocytic changes caused by oxidative membrane injury and impaired hemoglobin synthesis. Similarly, the elevation in platelet count may represent a compensatory inflammatory response to tissue injury induced by cisplatin toxicity. A recent systematic review by Frontiers in Pharmacology (2024) confirmed that platinum-based drugs, including cisplatin, cause hematological toxicity characterized by anemia, thrombocytopenia, leukopenia, and bone marrow dysfunction through oxidative stress and DNA damage (wang et al 2024).

In contrast, treatment with bioconjugated CuONPs (CuONPs-BCC) significantly improved these hematological parameters, as shown by the significant increase in RBC and HCT levels and the reduction in MCV and PLC compared with the cisplatin group. This improvement may be explained by the antioxidant and cytoprotective properties of copper nanoparticles conjugated with human serum albumin, which help neutralize free radicals, reduce oxidative stress, preserve erythrocyte membrane integrity, and support normal hematopoietic activity (Davoudi et al., 2022).The protective role of antioxidant therapy against cisplatin-induced toxicity has also been supported by recent studies, Hassan et al. (2022) demonstrated that antioxidant treatment significantly reduced cisplatin-induced tissue damage and improved physiological parameters by suppressing oxidative stress and inflammation.

Similarly, El-Shoura et al. (2024) reported that antioxidant agents protect against cisplatin toxicity through modulation of Nrf2 signaling and reduction of inflammatory pathways.

The BCC group also showed partial improvement in some parameters, but the effect was less pronounced compared with the CuONPs-BCC group, indicating that the nanoparticle bioconjugation with albumin enhanced the protective efficacy. Overall, these findings suggest that CuONPs-BCC may serve as a promising therapeutic strategy against cisplatin-induced hematological toxicity through its antioxidant, anti-inflammatory, and cytoprotective mechanisms.

6. Biochemical parameters

The present findings demonstrated a significant increase in blood glucose levels in the cisplatin-treated group compared with the control group. This elevation may be attributed to cisplatin-induced oxidative stress and metabolic disturbances, which impair insulin signaling and glucose metabolism. Cisplatin is known to enhance the generation of reactive oxygen species (ROS), leading to cellular damage and disruption of normal metabolic pathways (Mirzaei et al., 2021) . In contrast, treatment with CuONPs-BCC significantly reduced blood glucose levels compared with the cisplatin group, with values approaching normal levels. This protective effect may be related to the antioxidant and cytoprotective properties of nanoparticles and bioactive compounds present in BCC, which help reduce oxidative stress and restore metabolic balance (Alkhalaf et al., 2023). The BCC-treated group also showed an improvement in glucose levels, although the effect was less pronounced than that observed in the CuONPs-BCC group.

Regarding CRP results, the current study revealed a marked elevation in CRP levels in the cisplatin group compared with the control group, indicating the induction of a strong inflammatory response. Cisplatin-induced inflammation is closely associated with excessive ROS production and activation of inflammatory mediators (Han et al., 2023). However, administration of CuONPs-BCC significantly decreased CRP levels compared with cisplatin-treated rats, suggesting a potent anti-inflammatory activity. This effect may be explained by the ability of metal-based nanoparticles and natural bioactive compounds to suppress inflammatory pathways and reduce oxidative damage (Saif-Elnasr et al., 2023). Similarly, BCC treatment reduced CRP levels, but its anti-inflammatory effect remained weaker than that of CuONPs-BCC, indicating that nanoformulation enhanced the biological efficacy of the treatment.

The present findings showed a slight increase in IgG levels in the cisplatin-treated group compared with the control group. This increase may be attributed to the immune response induced by cisplatin-associated oxidative stress and inflammation. Cisplatin is known to stimulate immune reactions and inflammatory cytokine production, which may lead to elevated immunoglobulin synthesis, including

IgG. These findings are in agreement with the study of Zhao et al. (2022), who reported that cisplatin-induced oxidative stress can alter immune parameters and stimulate inflammatory immune responses. In contrast, the CuONPs-BCC-treated group recorded the highest IgG levels among all experimental groups, with a clear significant difference compared with the cisplatin group. This result suggests that CuONPs-BCC enhanced the immune response. Such an effect may be related to the antioxidant and immunomodulatory properties of copper nanoparticles and natural bioactive compounds, which help protect immune cells and improve their functional activity. Similar observations were reported by Han et al. (2023), who demonstrated that metal-based nanoparticles possess significant immunomodulatory and anti-inflammatory activities. Meanwhile, the BCC group showed IgG levels relatively close to those observed in the cisplatin group, indicating that its immunological effect was less effective than that of the nanoformulated CuONPs-BCC treatment. These findings are consistent with the study of Saif-Elnasr et al. (2023), who found that nanoformulations exhibit stronger protective and biological effects than crude natural compounds alone due to improved bioavailability and cellular interaction.

7. Histological analyzes

The histopathological examination showed that cisplatin caused severe structural damage in the studied organs, including necrosis, inflammatory infiltration, hemorrhage, and tissue disorganization (Ahmad et al., 2023). Cisplatin administration has been reported to alter physiological and metabolic functions, resulting in significant changes in organ weights and histopathological architecture. These alterations may be related to lipid peroxidation, inflammatory infiltration, and impairment of endogenous antioxidant defense systems such as glutathione and superoxide dismutase (Altalhi and al., 2023).

In the liver, cisplatin treatment induced severe histopathological alterations characterized by hepatocellular necrosis, hemorrhage, and inflammatory cell infiltration. The presence of inflammatory cells around damaged hepatocytes may indicate activation of inflammatory and immune responses following hepatic injury (Ahmad et al., 2023). After treatment with CuONPs-BCC, liver sections showed better preservation of hepatic architecture with reduced necrotic areas and less inflammatory infiltration, suggesting attenuation of liver damage and improvement of tissue recovery (Kundu, 2025).

Kidney sections from the cisplatin-treated group revealed marked glomerular atrophy, dilation of Bowman's space, tubular degeneration, necrosis, and inflammatory infiltration. The inflammatory infiltration observed in renal tissue may reflect recruitment of immune cells following cellular injury and tissue stress (Căruntu et al., 2021). In contrast, treatment with CuONPs-BCC improved the general

organization of renal tissue, reduced inflammatory infiltration, and preserved glomerular and tubular structures, indicating a protective effect against cisplatin-induced nephrotoxicity (He et al., 2024).

In the heart tissue of the cisplatin group showed degeneration of myocardial fibers together with collagen accumulation and disorganization of cardiac structure. Similar histopathological alterations associated with cytotoxicity and oxidative stress were reported by Morsy et al. (2021). Increased collagen deposition is commonly associated with chronic inflammation and tissue remodeling after cellular injury (Al-Bairuty et al., 2013). The inflammatory changes observed in cardiac tissue may also be related to infiltration of immune cells and activation of inflammatory pathways during tissue damage (Backman et al., 2021). Treatment with CuONPs-BCC reduced myocardial degeneration and collagen deposition while improving the arrangement of myocardial fibers, suggesting partial restoration of cardiac tissue integrity (Kundu, 2025).

In the spleen, cisplatin treatment caused disruption of the white pulp and red pulp together with necrotic changes and loss of normal splenic organization. Such alterations may indicate impairment of immune activity and disturbance of immune cell distribution (Markowski et al., 2022). Since the white pulp normally contains large populations of B lymphocytes (LB), T lymphocytes (LT), plasma cells, and NK cells, these structural changes may reflect inflammatory stress and immune imbalance (Căruntu et al., 2021; Backman et al., 2021). Similar disturbances in immune cell infiltration and tissue immune organization were described by Nederlof et al. (2019). After treatment with CuONPs-BCC, splenic tissue appeared more organized with reduced necrotic areas and partial restoration of the white pulp structure, suggesting improvement of immune tissue integrity and reduction of inflammatory damage (Kundu, 2025).

Conclusion

conclusion

In conclusion, cancer is one of the most prevalent diseases worldwide and can lead to death due to its severe complications. The traditional "one-compound–one-target" treatment approach has proven to be insufficient over time. Fortunately, recent research has demonstrated that multi-target therapies may inhibit several factors induced by chemotherapy, such as immunodepression in cancer patients, which contribute to the development and progression of cancer symptoms. Based on our findings, we were able to draw the following conclusions:

- ✓ The biosynthesis of copper nanoparticles (CuNPs) using human serum albumin (HSA) and their bioconjugation with snail breast cancer cells (BCC) highlighted their potential as natural nanoparticle stabilizers for the production of stable nanoparticles with very small crystallite sizes, approximately 8 nm for CuONPs and 8.3 nm for CuONPs-BCC. These findings were confirmed by various characterization techniques, including UV–Vis spectroscopy, FT-IR spectroscopy, XRD, and SEM/EDX analyses.
- ✓ The ***in-vitro*** study on **CuONPs** and **CuONPs-BCC** have shown significant antioxidant, anti-inflammatory and **cell membrane stabilization** properties, suggesting these nanoparticles could be valuable biological and medicinal agents for treating various diseases linked to oxidative stress and inflammation
- ✓ *In vivo* acute toxicity investigation gives permission for the safe use of CuONPs-BCC as therapeutic cancer vaccin.
- ✓ Cancer vaccine using CuONPs-BCC and BCC ameliorate the physiological parameters in our study, through enhancing the body weight, with reducing the relative liver, heart, spleen and kidneys weight that clearly backing the therapeutic efficacy against alteration of tissues due to cisplatin effect.
- ✓ Treatment with CuONPs-BCC and the BCC vaccine resulted in a significant improvement in both erythrocyte and leukocyte parameters in immunodepressed rats treated with cisplatin. This improvement was demonstrated by a reduction in anemia-related indices and modulation of the immune response, leading to a decrease in the immunological side effects induced by cisplatin.
- ✓ Increased immunoglobulin G levels after CuONPs-BCC vaccination is considered evidence of a successful immune response induced by the cancer vaccine.
- ✓ The positive impact of CuONPs-BCC and the BCC vaccine on restoring some of biochemical markers and through the beneficial effect against the alteration in various biological systems that linked to our studying data.

- ✓ Amelioration of oxidative stress markers demonstrate that CuONPs-BCC and BCC helped reduce lipid peroxidation (MDA levels) and improve antioxidant defenses through enhancing GSH levels and SOD activities in different organs, especially in the liver, kidney, heart, and spleen.
- ✓ Histopathological examination of the liver, kidney, heart, and spleen confirmed the previously mentioned findings, showing a noticeable improvement in tissue organization with reduced necrosis, inflammatory infiltration, and cellular degeneration compared with the cisplatin-treated group. These beneficial effects of CuONPs-BCC and the BCC vaccine may be attributed to their ability to improve antioxidant parameters and preserve tissue structure, suggesting that the vaccine could reduce cellular damage caused by cisplatin-induced oxidative stress and inflammation.
- ✓ ✓ The results also showed that combining CuONPs with BCC enhanced the biological effect of the formulation and provided better protection against cisplatin-induced toxicity compared with BCC alone.
- ✓ Overall, the findings of the present study suggest that CuONPs-BCC vaccine could be considered a promising therapeutic approach for reducing cisplatin-induced organ toxicity and protecting tissues from oxidative damage.

Perspectives

- ✓ Further studies are needed to better understand the molecular mechanisms involved in the protective effect of CuONPs-BCC, particularly those related to oxidative stress, inflammation.
- ✓ Additional investigations should be conducted to evaluate the long-term safety and biocompatibility of CuONPs-BCC before considering clinical applications.
- ✓ Future work may also focus on improving nanoparticle stability and targeting efficiency by combining CuONPs with other biological or polymeric materials.
- ✓ It would also be interesting to explore the potential use of CuONPs-BCC as a targeted drug delivery system in cancer therapy and other diseases associated with oxidative stress.
- ✓ More in vivo studies and advanced preclinical investigations are recommended to confirm the therapeutic efficiency of this nanosystem under different pathological conditions.

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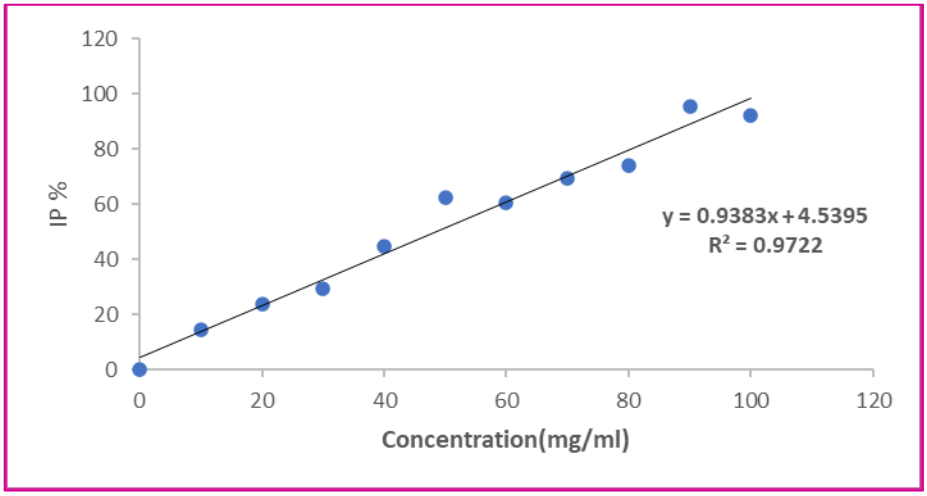
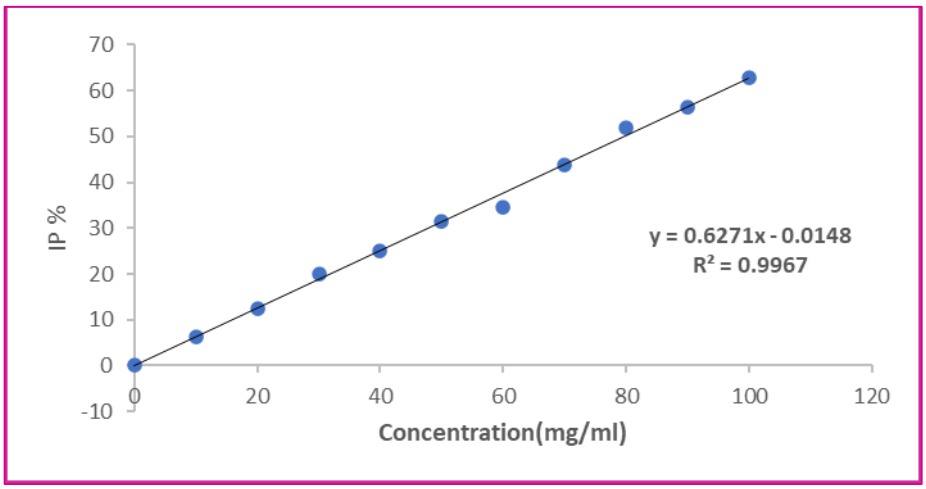
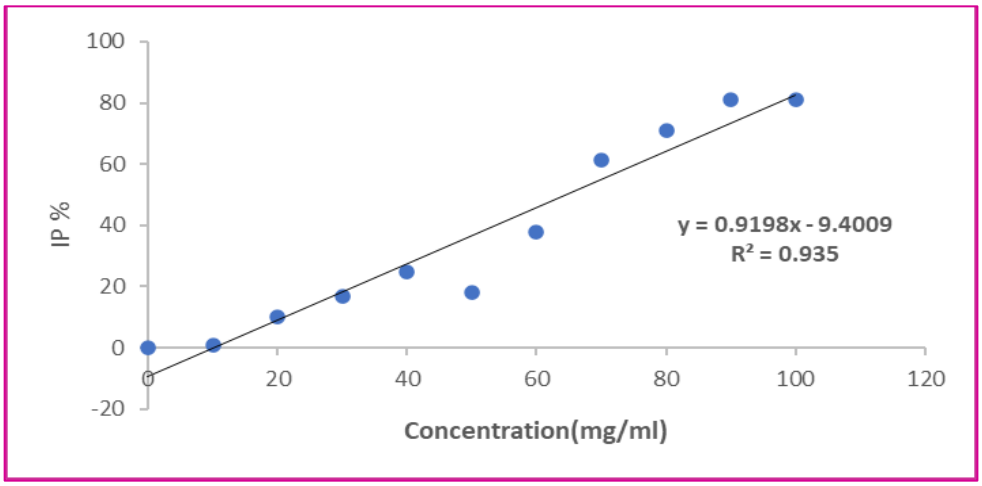
Annex



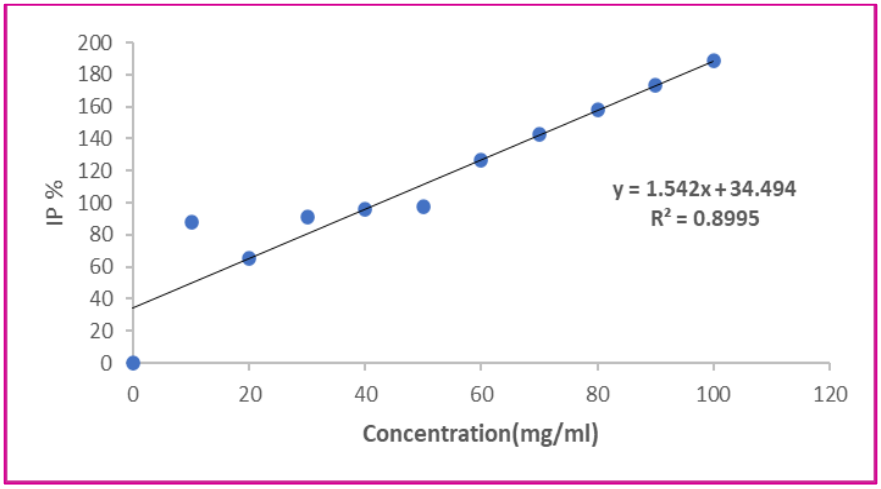
Annexe 1 : IP of FRAP assay in different concentrations of ascorbic acid.



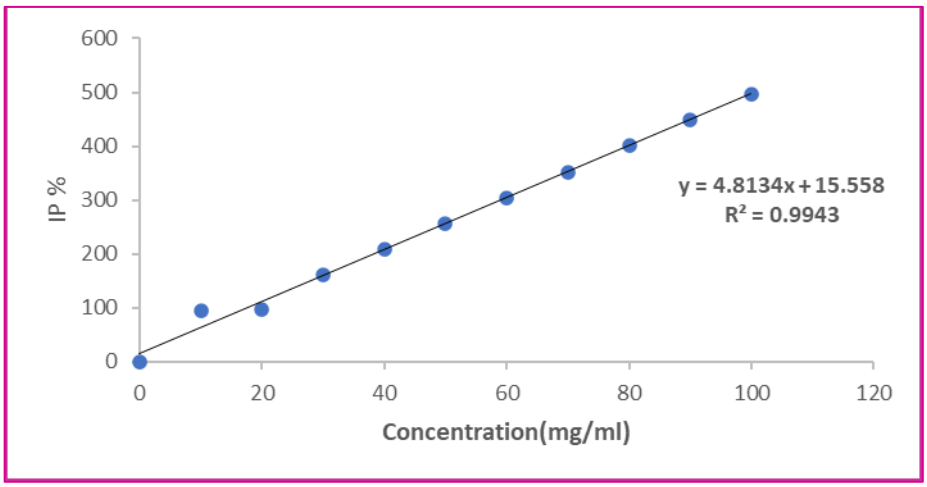
Annexe 2: IP of FRAP assay in different concentrations of CuONPs.



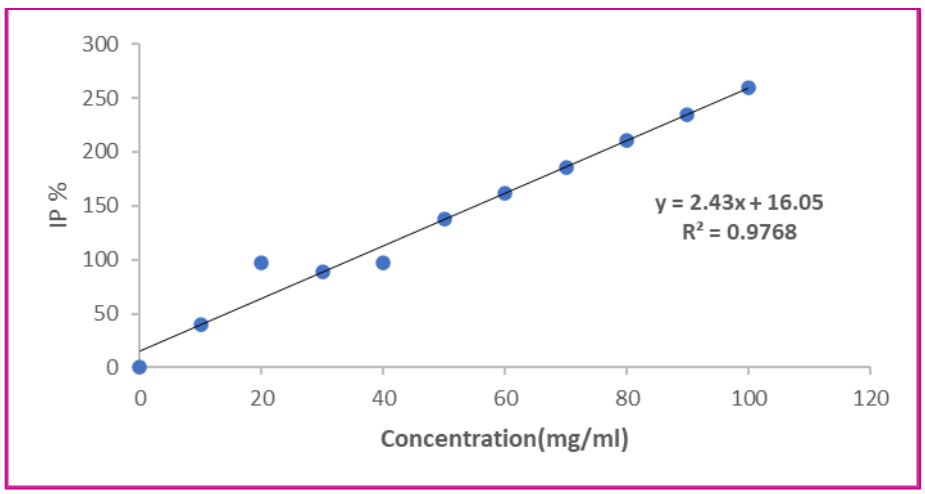
Annexe3 : IP of FRAP assay in different concentrations of CuONPs-BCC.



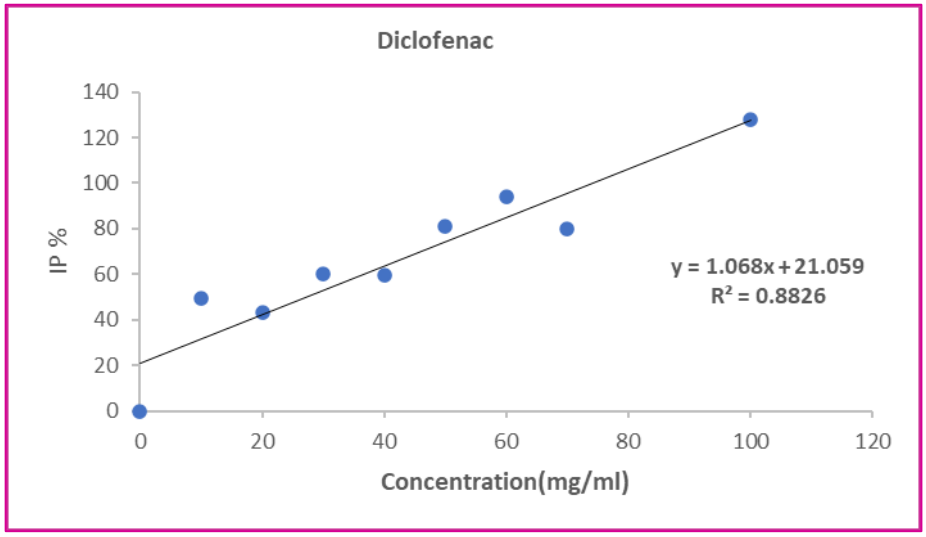
Annexe 4 : IP of Hemolysis assay in different concentrations of Diclofinac.



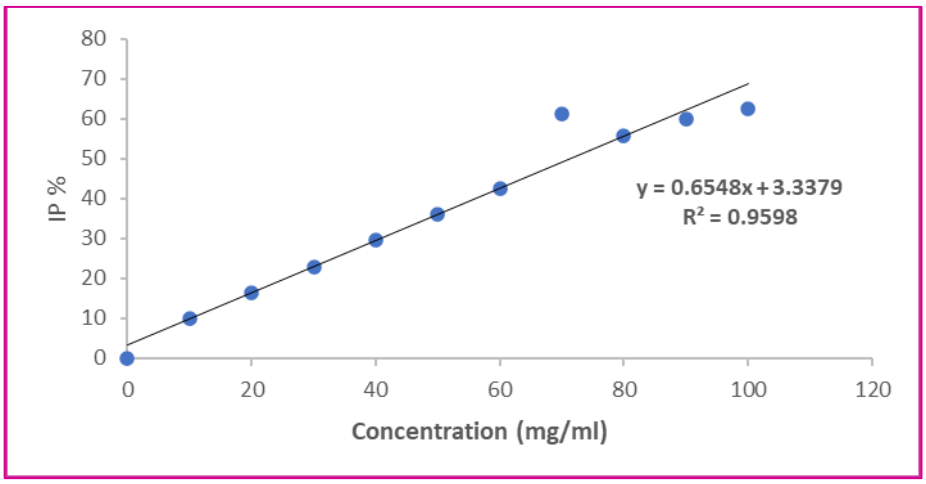
Annexe 5 : IP of Hemolysis assay in different concentrations of CuONPs



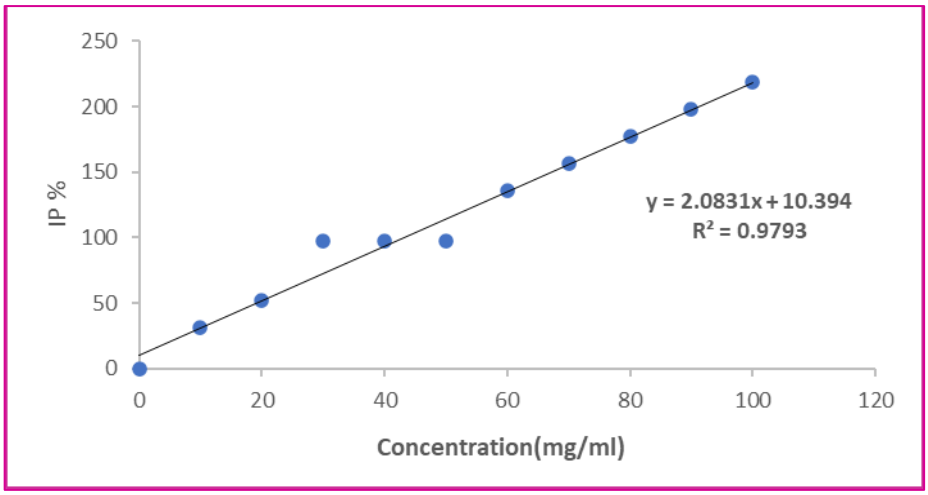
Annexe 6 : IP of Hemolysis assay in different concentrations of CuONPs-BCC.



Annexe 7: IP of Anti-inflamatory assay in different concentrations of Diclofinac.



Annexe 8: IP of Anti-inflamatory assay in different concentrations of CuONPs.



Annexe 9: IP of Anti-inflamatory assay in different concentrations of CuONPs-BCC.