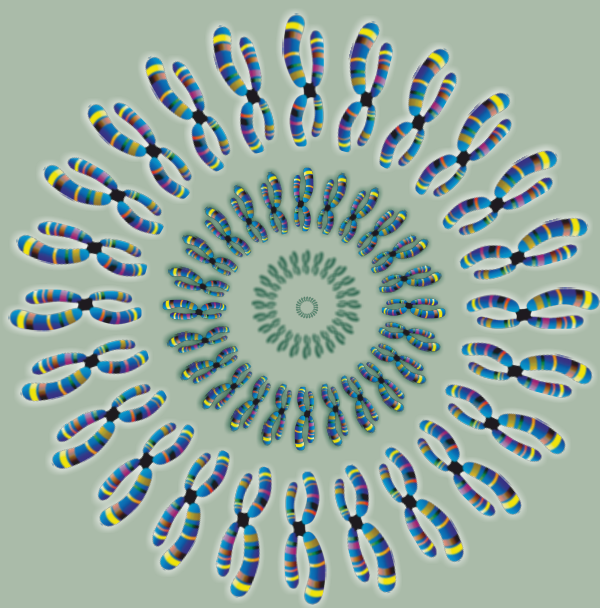
ALEXANDRU IOAN CUZA UNIVERSITY OF IASI

JOURNAL OF EXPERIMENTAL AND MOLECULAR BIOLOGY

Volume 26 Number 4 (2025)

ISSN 2601 – 6974



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„ALEXANDRU IOAN CUZA” UNIVERSITY OF IAȘI

JOURNAL OF EXPERIMENTAL AND MOLECULAR BIOLOGY

TOME XXVI, Number 4 **2025**

Editura Universității „ALEXANDRU IOAN CUZA” din Iași

EDITORIAL OFFICE

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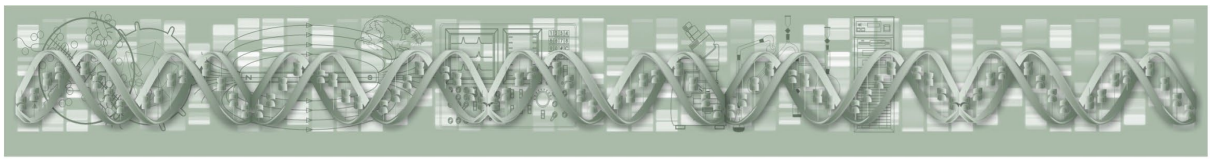

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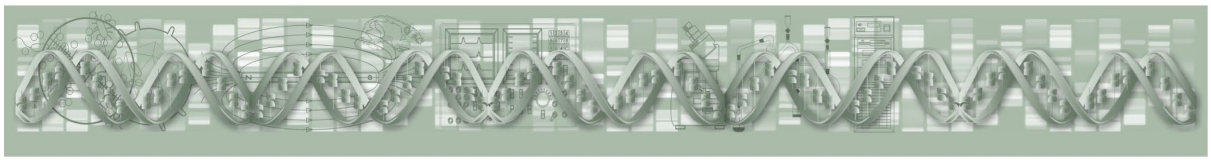

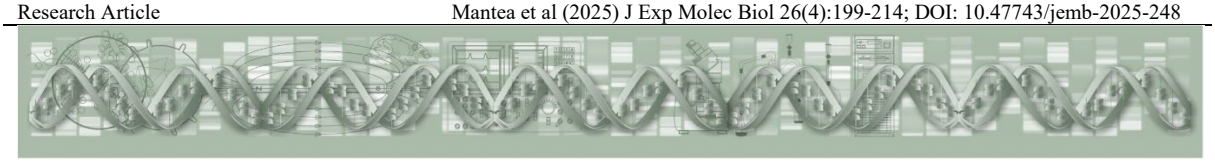

UNRAVELING THE PLANT GROWTH-PROMOTING POTENTIAL OF *BACILLUS SAFENSIS* P1.5S THROUGH GENOME ANALYSIS

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Abstract

Plant growth-promoting bacteria have emerged as promising eco-friendly alternatives to traditional agricultural practices. These beneficial microbes promote plant growth through various mechanisms including nitrogen fixation, production of indole-3-acetic acid (IAA), salicylic acid (SA), volatile organic compounds (VOCs), and antimicrobial secondary metabolites. In this study, we performed a genome-based characterization of bacterial strain P1.5S using bioinformatic tools to identify genes associated with plant growth promotion. The draft genome of strain P1.5S is 3,667,318 bp in size, assembled into 13 contigs. Taxonomic analysis confirmed the identity of the strain as *Bacillus safensis* (dDDH: 80.6%; ANI: 97.84%). Our *in silico* investigation revealed gene clusters related to nitrogen fixation (10 genes), as well as genes involved in the production of IAA (12 genes), SA (7 genes) and VOCs biosynthesis. Additionally, the genome encodes biosynthetic gene clusters for secondary metabolites with antimicrobial properties such as lipopeptides, peptides and polyketides. The presence of genes related to siderophore and hydrolytic enzymes production highlights the strain's potential for biocontrol. Moreover, genes associated with root colonization further support the plant-beneficial potential of this strain. *Bacillus safensis* P1.5S is a promising candidate for agricultural practices, but further greenhouse and field studies are necessary to validate its potential.

Keywords: draft genome, *Bacillus safensis* P1.5S, plant growth promoting traits, nitrogen fixation, phytohormone production, biocontrol

Introduction

One of the greatest challenges of the 21st century is meeting the food demands of a growing global population, while preserving the environment and maintaining biodiversity. Historically, food production has relied heavily on the use of chemical fertilizers, which significantly increased crop productivity. However, considerable environmental drawbacks related to this practice were evidenced, including water contamination, soil degradation and loss of biodiversity (Pahalvi et al. 2021). Given the negative impact of excessive chemical fertilizer use, there is an increasing need for innovative solutions and more sustainable agricultural practices to help farmers manage their fields more efficiently (Saleem et al. 2023).

Plant growth-promoting bacteria (PGPB) have emerged as a promising solution to enhance crop yields and sustainability, providing a more eco-friendly alternative to conventional agricultural methods (Katsenios et al. 2022). These bacteria colonize the rhizosphere of plants and promote growth through both direct and indirect mechanisms, including biological nitrogen fixation

Received 18 August 2025; Reviewed 14 October 2025; Accepted 15 October 2025;
Published Online 23 October 2025; Published 23 December 2025

(BNF), solubilization of phosphorus compounds, production of indole-3-acetic acid (IAA), salicylic acid (SA), siderophores and volatile organic compounds (VOCs), activity of 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase), and microbial antagonism (Kumar et al. 2022). In addition, it has been shown that PGPB improves crop tolerance to abiotic stress, as well as enhance their growth and quality characteristics. Bacteria such as *Rhizobium*, *Azospirillum*, *Bacillus*, and *Pseudomonas* have been utilized as effective plant growth promoters (Katsenios et al. 2022). Several benefits of PGPB agricultural use were outlined, including environmental sustainability, improved soil health, nutrient availability and fixation, enhanced plant growth and productivity, and reduction of soil and water contamination (Souza et al. 2015).

In this study, we isolated a *Bacillus safensis* strain (named P1.5S), from a phosphorus-deficient soil. *In vitro* tests revealed that the P1.5S strain possesses several traits involved in plant growth promotion, including phosphorus solubilization, production of IAA, siderophores, ACC deaminase and antimicrobial lipopeptides. It is also a stress-tolerant bacterium, capable of solubilizing phosphorus under pH, salt, and temperature stress (Mantea et al. 2025). Additionally, *Bacillus safensis* P1.5S forms a robust biofilm, which plays a crucial role in root colonization. Here, we report the draft genome sequence of *B. safensis* P1.5S, providing important insights into the genes involved in the plant growth-promoting capabilities. Our results highlight the potential of P1.5S strain to be used as a biofertilizer in agricultural applications. Nonetheless, additional research is required to evaluate the strain's performance under field conditions.

Materials and Methods

Isolation of *Bacillus safensis P1.5S* and genomic DNA sequencing

The strain used in this study was isolated from phosphorus-deficient soil cultivated with maize located in the north-eastern part of Iasi County, Romania. The soil sample was serially diluted with distilled sterile water, and 50 µL from the suspension were plated onto Pikovskaya (PVK) medium containing insoluble $Ca_3(PO_4)_2$ (TCP) as the sole phosphorus source. After incubation at 28 °C for 7 days, a small, bright yellow colony was selected based on the formation of a clear zone around it (indicating TCP solubilization) and inoculated into 10 mL Luria-Bertani (LB) broth. Following a 14-hours incubation at 28 °C, 9 mL of the culture was centrifuged, and the pellet was used for genomic DNA extraction using the DNeasy UltraClean Microbial Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The whole genome *de novo* sequencing was performed using the Illumina NovaSeq 6000 (Macrogen Europe BV, Amsterdam, Netherlands), and library preparation was conducted with the TruSeq DNA PCR-Free Kit, generating paired-end 151 nt reads.

Genome assembly, annotation and taxonomic identification

First, the obtained raw data was assessed for read quality using FastQC v0.11.9 (Andrews 2010) and filtered with Fastp v0.23.2 (Chen et al. 2018) to remove the adapters and low-quality sequences. *De novo* genome assembly of the resulting filtered reads was performed using Unicycler v0.4.9 (Wick et al. 2017). The quality and contiguity statistics of the assemblies were evaluated using QUAST v5.2.0 (Gurevich et al. 2013), while completeness and contamination were assessed using CheckM v1.2.2 (Parks et al. 2015). The draft genome was uploaded to the NCBI Prokaryotic Genome Annotation Pipeline (PGAP v6.5) for automatic annotation (Tatusova et al. 2016). In addition, the draft genome was submitted to TYGS (Type (Strain) Genome Server) for taxonomic identification (Meier-Kolthoff and Göker 2019) and to antiSMASH 7.0 for the prediction of secondary metabolite biosynthesis gene clusters (Blin et al. 2023). The TBLASTN algorithm (Altschul et al. 1990) was used to search for genes related

to plant growth promotion, chemotaxis, motility and biofilm formation. Genes with an identity higher than 30% and a query coverage value above 70% were considered positive hits. A database of UniProt protein sequences of interest was created and subsequently searched against the draft genome (The UniProt Consortium, 2025). For all tools and servers, default parameters were used unless otherwise stated.

Results and discussions

Genomic characterization of *Bacillus safensis* P1.5S

The sequencing runs allowed the acquisition of 13.6 million reads totaling 2.0 Gbp. After filtering > 91% of the reads had Q scores > 30 and an average Phred score/read of 36. A total of 13.47 million reads were used for the genome assembly. The draft genome assembly has a length of 3,667,318 bp and consists of 13 contigs, with an N50 of 975,127 bp and a C+G content of 41.5% (Table 1). The CheckM analysis indicated a completeness of 99.22% with minimal contamination (0.29%). According to the functional annotation performed by NCBI PGAP, a number of 3,756 genes, of which 3,667 genes are protein-coding sequences, were identified (Table 1).

Table 1. *Bacillus safensis* P1.5S genome features

Attribute	Value
Total reads (before filtering)	13,617,686
Total reads (after filtering)	13,475,572
Genome coverage (×)	530
Genome size (bp)	3,667,318
Genes (total)	3756
CDSs (total)	3705
CDSs (with protein)	3667
Number of contigs	13
Contig N50 (bp)	975,127
Contig L50	2
GC content (%)	41.5
Genes (RNA)	51
tRNA	44
rRNA	1, 1 (16S, 23S)
ncRNA	5
Pseudogenes	38
Accession number	JARZFW000000000

CDS – coding sequence; GC – guanine-cytosine content; ncRNA – non-coding RNA; rRNA – ribosomal RNA; tRNA – transfer RNA.

Phylogenomic analysis of *Bacillus safensis* P1.5S

Based on TYGS analysis, a phylogenetic tree divided into two clades with a common ancestor was constructed - Figure 1. The first clade is divided into two branches. One of the branches contains P1.5S strain along with *Bacillus safensis* subsp. *osmophilus* CECT 9344T, *B. safensis* FO-36b, *B. australimaris* NH7I_1 and two strains of *B. pumilus* (ATCC 7061 and NCTC 10337); the other branch consists of *B. zhangzhouensis* MCCC 1A08372. The P1.5S strain is clustered along with *B. safensis* FO-36b and *B. safensis* subsp. *osmophilus* CECT 9344T in a unique branch. TYGS analysis also revealed a digital DNA-DNA hybridization (dDDH) value of 80.6% when compared to *Bacillus safensis* subsp. *osmophilus* CECT 9344T and 70.7% when compared to *Bacillus safensis* FO-36b (Table 2). The dDDH values are above the common

threshold accepted for species-level delineation (70%) (Meier-Kolthoff and Göker 2019), suggesting a high similarity between P1.5S strain and *B. safensis* subsp. *osmophilus* CECT 9344T/*Bacillus safensis* FO-36b. Based on these results we concluded that P1.5S strain belongs to *Bacillus safensis* species. Also, the average nucleotide identity (ANI) of the P1.5S strain genome was compared with that of *Bacillus safensis* subsp. *osmophilus* CECT 9344T and *Bacillus safensis* FO-36b, yielding values of 97.84% and 96.59%, respectively. These high ANI values confirm the close genetic similarity between the P1.5S strain and the two *Bacillus safensis* strains. In support of our conclusion, the G+C content difference between the P1.5S strain and *Bacillus safensis* subsp. *osmophilus* CECT 9344T (0.6%) and *Bacillus safensis* FO-36b (0.11%) falls within the 1% variation range typically considered for strains of the same species (Palma et al. 2024).

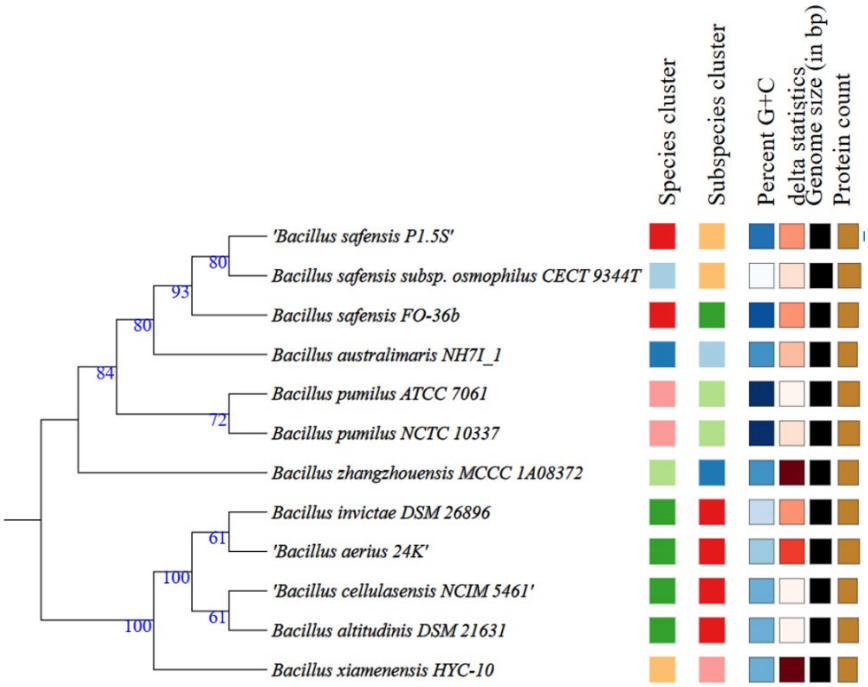

Figure 1. Phylogenetic tree based on the whole genome of P1.5S strain. Tree inferred with FastME 2.1.6.1 from GBDP distances calculated from genome sequences. The branch lengths are scaled in terms of GBDP distance formula d_5. The numbers are GBDP pseudo-bootstrap support values > 60 % from 100 replications, with an average branch support of 81.2 %. The tree was rooted at the midpoint.

Table 2. Pairwise dDDH comparisons between P1.5S strain genome and the type-strain genomes selected by TYGS. The dDDH values, their confidence intervals for the d4 formula and the G+C content difference are shown.

Subject strain	dDDH (d4 in %)	C.I. (d4 in %)	G+C content difference (in %)
Bacillus safensis subsp. *osmophilus* CECT 9344T	80.6	(77.7 - 83.2)	0.6
Bacillus safensis FO-36b	70.7	(67.7 - 73.6)	0.11
Bacillus australimaris NH7I 1	52.3	(49.7 - 55.0)	0.15
Bacillus pumilus NCTC 10337	45	(42.4 - 47.6)	0.22
Bacillus pumilus ATCC 7061	44.9	(42.4 - 47.5)	0.17
Bacillus zhangzhouensis MCCC 1A08372	42.1	(39.6 - 44.7)	0.11

Bacillus xiamenensis HYC-10	37.5	(35.1 - 40.0)	0.2
Bacillus invictae DSM 26896	36.6	(34.2 - 39.2)	0.39
Bacillus aerius 24K	36.6	(34.1 - 39.1)	0.28
Bacillus altitudinis DSM 21631	36.6	(34.1 - 39.1)	0.23
Bacillus cellulasensis NCIM 5461	36.5	(34.0 - 39.0)	0.17

dDDH = digital DNA-DNA hybridization calculated using d4 formula (Meier-Kolthoff and Göker 2019)
C.I. = confidence intervals

Prediction of plant growth promotion traits

Several genes involved in plant growth promotion were detected in *B. safensis* P1.5S genome (Table 3). P1.5S strain harbors genes important for agricultural applications, influencing plant growth through both direct and indirect mechanisms. Thus, 10 genes related to nitrogen fixation such as *nifU*, as well as several nitrogen metabolism-related genes including those responsible for nitrogen transport (*gltP*, *amtB*/*nrgA*, and *nrgB*/*glnK*), nitrogen assimilation (*glnA*, *glnH* and *glnR*), nitrate reduction (*nasE*, *nasD*) and glutamate metabolism (*gltX*) were identified (Hazarika et al. 2023). These findings suggest a putative direct role of the P1.5S strain in plant growth promotion by increasing nitrogen availability, thereby enhancing photosynthesis, improving yields, and contributing to stress tolerance (Vats et al. 2021). Additionally, the P1.5S strain may improve soil nitrogen availability, potentially reducing the reliance on chemical fertilizers (Timofeeva et al. 2023).

Table 3. Genes involved in plant growth promotion putatively encoded by the *Bacillus safensis* P1.5S genome

NCBI Gene ID*	UniProt Gene name	UniProt Protein ID*	Identity (%)	Query coverage (%)	E-value	Total score
Nitrogen fixation and metabolism						
3026303	***nifU***	Q631Z1	83.45	97	2e-77	246
938421	***gltP***	P39817	71.43	100	0.0	1011
			40.53	97	2e-93	305
			39.02	97	8e-90	294
936862	***gltX***	P22250	82.82	100	0.0	845
940070	***glnR***	P37582	81.48	99	1e-70	226
940020	***glnA***	P12425	94.82	100	0.0	880
938139	***glnH***	O34563	68.27	99	4e-113	432
			31.46	96	7e-29	112
936937	***nrgB***	Q07428	84.07	97	2e-61	199
936933	***nrgA***	Q07429	73.38	100	0.0	592
938331	***nasE***	P42436	64.36	95	2e-41	141
938329	***nasD***	P42435	68.83	100	0.0	1702
IAA production						
939011	***trpA***	P07601	71.16	100	9e-129	399
939010	***trpB***	P07600	80.40	100	0.0	674
939004	***trpC***	P03964	69.92	94	1e-104	329
939007	***trpD***	P03947	57.35	100	2e-118	373
939008	***trpE***	P03963	68.50	99	0.0	842
			38.24	92	7e-86	287
939006	***trpF***	P20167	51.38	99	2e-70	229

939361	***trpS***	P21656	85.11	100	0.0	584
939963	***dhaS***	O34660	79.19	100	0.0	1375
			42.29	96	2e-125	567
			37.04	92	3e-88	293
			34.55	96	5e-73	486
937252	***ywdH***	P39616	65.43	100	0.0	800
			30.80	93	8e-53	471
			31.67	88	1e-50	182
937464	***ysnE***	P94562	61.59	100	4e-60	197
936875	***ywkB***	P45869	72.19	95	7e-137	425
938351	***amhX***	P54983	65.16	98	2e-167	516
Salicylic acid production						
939014	***aroA***	P20691	76.06	100	0.0	650
939001	***aroB***	P31102	59.72	99	6e-156	482
938963	***aroC***	P35146	31.84	96	6e-41	147
939000	***aroF***	P31104	82.05	100	0.0	643
937820	***aroE***	P54374	67.14	100	3e-129	401
			33.56	96	6e-47	165
939005	***aroH***	P19080	69.29	100	1e-57	189
937190	***menF***	P23973	55.77	99	2e-174	540
Siderophores production and iron transport						
936592	***besA***	O32102	49.10	97	6e-86	335
936579	***dhbA***	P39071	55.94	100	6e-95	490
			33.98	97	2e-38	546
			30.23	97	5e-21	89.4
937162	***dhbC***	P45744	58.40	100	6e-153	475
936582	***dhbE***	P40871	70.94	96	0.0	763
936582	***dhbB***	P45743	54.81	100	8e-114	358
936569	***dhbF***	P45745	59.63	100	0.0	2932
			31.48	89	0.0	2343
			31.75	90	0.0	2402
			32.81	94	0.0	3400
936706	***sufB***	O32162	95.05	100	0.0	1096
938871	***sufD***	O32165	81.01	100	0.0	933
Volatile organic compounds						
936852	***alsS***	Q04789	66.97	98	0.0	771
936852	***alsR***	Q04778	58.74	95	2e-117	534
939490	***bdhA***	O34788	79.30	100	3e-180	768
936152	***acoA***	O31404	73.99	96	3e-168	516
			49.21	98	5e-98	590
939697	***acoB***	O34591	80.18	99	0.0	572
			47.76	98	7e-99	911
Hydrolytic enzymes						
939313	***aprE***	P04189	62.04	100	1e-141	442
			50.00	98	1e-113	653
			37.89	97	3e-61	333
938861	***lipA***	O32129	97.32	100	0.0	656

* Gene IDs and protein IDs are derived from the genome of the reference strain *Bacillus subtilis* 168.

Percent identity (%) is the percentage of nucleotides that are identical between the genome of the bacterial strain and the sequence of interest.
Query cover (%) is the percentage of the query sequence (strain genome) overlapping the reference sequence.
E value is a parameter that describes the number of hits that can be "expected" to be found by chance when searching a database of a given size. The lower the E-value, the better the match.

Genome analysis revealed the presence of several genes that contribute to production of two key plant hormones – IAA (12 genes) and SA (7 genes). Some genes are directly involved in the biosynthesis of tryptophan, a precursor to IAA (*trpA* to *trpF* genes) (Natori et al. 1990), while others are related to IAA biosynthesis or the regulation of IAA levels in plants (*dhaS*, *ywdH*, *ysnE*, *ywkB*, and *amhX* genes) (Shao et al. 2015). *aroA*, *aroB*, *aroC*, *aroE* and *aroH* genes play a direct role in the production of SA and *aroF* contributes to the production of phenylalanine, a precursor for SA (Polen et al. 2005). By producing IAA and SA, *Bacillus safensis* P1.5S may promote plant growth by enhancing cell elongation, division, and differentiation, stimulating root formation, improving germination and seedling growth, and modulating responses to environmental factors (Hayat et al. 2010).
Several genes associated with siderophore production and the assembly of iron-sulfur clusters such as *dhbA*, *dhbB*, *dhbC*, *dhbE*, *dhbF, besA* (involved in bacillibactin synthesis) or *sufB*, *sufD* (associated with the iron-sulfur cluster assembly pathway) were detected in *B. safensis* P1.5S genome (Blahut et al. 2020). By producing siderophores capable of binding, extracting and transporting iron near the plant roots, soil bacteria play an important role in iron acquisition, stimulating plant development and health (Leal et al. 2021).
The release of VOCs can directly influence plant growth by enhancing nutrient availability. Our data sustains the presence of *alsS*, *alsR*, *bdhA*, *acoA*, and *acoB* genes involved in the biosynthesis of acetoin and 2,3-butanediol, two potent volatile organic compounds that play significant roles in plant growth promotion (Altimira et al. 2022). Moreover, the presence of the *aprE* and *lipA* genes, encoding an alkaline protease (Jan et al. 2000) and a lipase respectively (Akatsuka et al. 1994), suggests that *Bacillus safensis* P1.5S may contribute to nutrient release and availability, root development, and plant stress tolerance. In addition, several genes involved in phosphorous solubilization, encoding the biosynthesis of organic acids, such as gluconic, formic, malic, citric, lactic, acetic, and succinic acids, or the synthesis of acid and alkaline phosphatases were also predicted in P1.5S genome (Mantea et al. 2025). These results highlight the potential of *Bacillus safensis* P1.5S to promote plant growth through direct mechanisms, including enhanced uptake of essential nutrients such as nitrogen, phosphorus, and iron, regulation of root development, cell elongation and division, and improved stress tolerance.
Regarding the indirect mechanisms, antiSMASH analysis predicted 12 biosynthetic gene clusters involved in the synthesis of secondary metabolites (Table 4). Eight of these clusters showed similarity to known compounds, including lipopeptides (fengycin, surfactin, and lichenysin production), peptides (bottromycin A2), polyketides (bacilysin), and siderophores (bacillibactin and schizokinen). These compounds exhibit a wide range of biological activities: antifungal and antibacterial (fengycin, bottromycin A2, bacilysin bacillibactin) (Coutte et al. 2010, Özcengiz and Öğülür 2015, Franz et al. 2021, Chakraborty et al. 2022), antibiofilm (lichenysin) (Yeak et al. 2022) and biosurfactant properties (surfactin) (Coutte et al. 2010). In addition, identification of genes involved in the production of siderophores, hydrolytic enzymes or VOCs (Table 3) highlights the potential role of *B. safensis* P1.5S in the biocontrol and pathogen defense, induction of systemic resistance, and improvement of plant health and stress tolerance (Chen et al. 2007). Many of the secondary metabolites identified in the P1.5S genome have also been reported in other *Bacillus safensis* strains (Li et al. 2021, Mateus et al. 2021).

Table 4. Biosynthetic gene clusters predicted in the *Bacillus safensis* P1.5S genome using antiSMASH

Contig	Region	Type	From (nt)	To (nt)	Most similar known cluster	Similarity (%)
JARZFW010000001	Region 1.1	betalactone	616,475	644,887	fengycin	53
	Region 1.2	terpene	714,926	736,800	bottromycin A2	6
	Region 1.3	Type III PKS	777,306	818,406	-	-
	Region 1.4	betalactone	1,270,349	1,302,80	-	-
JARZFW010000002	Region 2.1	other	632,972	674,393	bacilysin	85
	Region 2.2	NRP-metallophore/ NRPS	897,950	949,678	bacillibactin/ bacillibactin E/bacillibactin F	80
JARZFW010000003	Region 3.1	NI-siderophore/ terpene	111,749	149,400	schizokinen	60
	Region 3.2	RRE-containing	292,542	313,447	-	-
JARZFW010000005	Region 5.1	RiPP-like	27,719	38,045	-	-
	Region 5.2	NRPS	179,437	208,163	lichenysin	50
JARZFW010000006	Region 6.1	NRPS	1	44,033	surfactin	39
JARZFW0100000010	Region 10.1	NRPS	1	10,479	lichenysin	14

PKS – Poliketide synthetase;
NRPS – Non-ribosomal peptide synthetase;
NI-siderophore – NRPS-independent;
NRP-metallophore – Non-ribosomal peptide metallophores;
RRE-containing – Regulatory RNA Element;
RiPP-like - Other unspecified ribosomally synthesised and post-translationally modified peptide product.

Genes involved in root colonization predicted in *Bacillus safensis* P1.5S genome

According to BLAST analysis, the draft genome of *B. safensis* P1.5S contains several genes associated with plant root colonization capabilities (Table 5). First, the genome encodes at least 20 genes responsible for chemotaxis and motility, such as *mcpA*, *mcpB*, and *mcpC* involved in eliciting a response to changes in the concentration of environmental attractants and repellents (Müller et al. 1997, Liu et al. 2023). Like many *Bacillus* species, P1.5S strain may use a chemosensory pathway involving a histidine kinase (*cheA*) and a response regulator (*cheY*) to transmit signals from receptors to the flagellar motors, and *motA* and *motB* to encode membrane proteins that form the bacterial flagellar motor, essential for flagellar rotation and motility (Vats et al. 2021).

Four genes involved in the *quorum sensing* (QS) signaling system - critical for effective root colonization - were identified in the draft genome. These include *luxS* which is responsible for

the synthesis of autoinducer-2 (AI-2), a universal signaling molecule used in interspecies communication (Lombardía et al. 2006); *comP* and *comA* which encode a protein kinase and a response regulator protein, forming the two-component ComP-ComA signal transduction system essential for QS (Wolf et al. 2015); and *comQ* which is involved in the post-translational modification and export of the ComX quorum-sensing pheromone. This pheromone regulates key population-dependent processes that are important for the strain's ability to colonize plant roots and promote plant growth (Kalamara et al. 2018).

Moreover, 47 genes related to exopolysaccharide (EPS) production, biofilm formation and regulation were identified in *B. safensis* P1.5S genome. *TasA* is a part of the *tapA-sipW-tasA* operon and encodes a major protein component that forms functional, amyloid-like, protease-resistant fibers on hydrophobic biofilm surfaces. TapA is an accessory protein that facilitates the assembly of TasA fibers, while *bslA (yuaB)* is a hydrophobin that coats the biofilm surface, conferring hydrophobic properties (Zhang et al. 2022). The *epsA-epsO* operon is involved in EPS biosynthesis, which is essential for bacterial cell attachment to surfaces (Cámara-Almirón et al. 2020). The *remA* gene regulates extracellular polymeric matrix biosynthesis and biofilm formation by activating transcription of the matrix biosynthesis operons, including *tapA-sipW-tasA* (Hoffmann et al. 2021). The genus *Bacillus* exhibits a complex system for regulating biofilm formation, particularly under unfavorable environmental conditions (Omer Bendori et al. 2015). The activity of the two primary biofilm-forming operons (*tapA-sipW-tasA* and *epsA-O*) is indirectly regulated by the *spo0A* gene. In general, these histidine - kinase sensors detect signaling triggered by surfactin, a cyclic lipopeptide that is considered an auto-trigger of biofilm formation in many *Bacillus* species (Omer Bendori et al. 2015, Cámara-Almirón et al. 2020). In the case of P1.5S strain, surfactin may be synthesized by an enzyme complex encoded by the *srfAA - srfAD* gene cluster, with surfactin-triggered signaling activating Spo0A, leading to the formation of its phosphorylated form, Spo0A~P.

Table 5. Genes involved in chemotaxis, motility, *quorum sensing* and biofilm formation putatively encoded by the *Bacillus safensis* P1.5S genome

NCBI Gene ID*	UniProt Gene name	UniProt Protein ID*	Identity (%)	Query coverage (%)	E-value	Total score
939600	***cheA***	P29072	71,64	99	0	1009
940124	***cheB***	Q05522	62,01	99	1e-144	627
939621	***cheC***	P40403	74,76	98	2e-100	353
939003	***cheR***	P31105	71,21	100	5e-122	380
939239	***cheV***	P37599	73,51	99	2e-148	504
940120	***cheY***	P24072	91,67	100	7e-70	638
			36,97	99	4e-22	241
			33,63	99	9e-19	129
			33,63	93	2e-15	68,6
			31,86	93	1e-14	65,9
939957	***cheW***	P39802	70,20	96	3e-64	209
938840	***tlpA***	P39216	52,83	95	0	2638
937152	***tlpB***	P39217	52,73	99	0	2651
937154	***mcpA***	P39214	53,18	100	0	2672
937155	***mcpB***	P39215	53,31	100	0	2754
936206	***mcpC***	P54576	49,01	99	0	744

936102	***yfmS***	O06477	34,75	90	2e-44	158
			34,45	87	2e-42	323
			34,36	94	1e-35	656
939302	***motA***	P28611	69,70	97	6e-119	371
939304	***motB***	P28612	58,73	96	5e-92	293
51992978	***swrAA***	O32266	83,76	100	3e-63	204
939618	***swrB***	P40405	37,91	95	2e-28	107
14768252	***swrC***	O31501	66,62	95	0	1347
8303013	***swrD***	C0H412	74,65	100	8e-31	109
936739	***flgM***	P39809	52,27	100	1e-23	89,7
			***Quorum sensing* system**			
937106	***luxS***	O34667	89.17	100	4e-96	300
937179	***comA***	P14204	71.96	100	5e-104	326
			30.88	94	5e-24	133
938866	***comP***	Q99027	45.03	100	0	799
937180	***comQ***	P33690	40.66	90	2e-60	205
			Biofilm formation			
937956	***remA***	Q7WY72	95.51	100	5e-53	174
938655	***spo0A***	P06534	86.94	100	3e-157	724
938532	***tapA***	P40949	37.06	65	1e-33	125
938545	***tasA***	P54507	62.40	90	4e-99	314
938868	***sigW***	Q45585	90.91	100	1e-113	352
			31.06	85	2e-19	82,8
938544	***sinR***	P06533	93.69	100	1e-65	211
			45.05	99	8e-23	88.6
938543	***sinI***	P23308	43.75	84	2e-10	50.4
937009	***abrB***	P08874	93.75	100	2e-54	248
			53.26	95	8e-30	108
			42.86	94	2e-22	87.4
14769325	***ymcA***	O31779	83.92	100	4e-76	243
14769122	***ylbF***	O34412	74.81	87	1e-65	213
938482	***sigD***	P10726	88.98	100	3e-154	543
936362	***sigL***	P24219	52.15	95	2e-143	449
938582	***epsA***	P71050	40.44	96	1e-57	194
938640	***epsB***	P71051	64.18	87	1e-21	101
938571	***epsC***	P71052	60.27	99	0	718
938611	***epsD***	P71053	52.52	98	5e-135	423
938633	***epsE***	P71054	61.60	96	4e-110	446
938631	***epsF***	P71055	52.80	97	1e-130	456
937071	***epsG***	P71056	62.94	100	1e-151	470
938630	***epsH***	P71057	44.44	99	5e-98	442
937256	***epsI***	P71058	59.82	92	7e-135	421
937131	***epsJ***	P71059	33.23	94	2e-49	304
938240	***epsK***	P71060	41.63	99	3e-124	397
936365	***epsL***	P71062	65.31	97	4e-89	405
936372	***epsM***	P71063	48.28	93	1e-58	196
936364	***epsN***	Q795J3	64.42	95	2e-162	502
936731	***csrA***	P33911	81.36	79	2e-29	105
938831	***bslA***	P71014	66.18	73	2e-57	191

936830	***capA/pgsA***	P96738	59.10	99	3e-149	464
936833	***capB/pgsB***	P96736	87.02	100	0	716
936840	***capC/pgsC***	P96737	81.08	99	4e-72	231
936209	***mnaA***	P39131	80.37	99	0	630
938302	***yczE***	O34927	59.07	100	8e-75	242
936293	***ecsA***	P55339	81.56	98	1e-134	824
			32.61	91	3e-29	194
939298	***ecsB***	P55340	56.78	97	2e-155	483
939774	***ecsC***	P55341	55.31	95	4e-75	244
938306	***srfAA***	P27206	53.15	94	0	6197
			38.70	99	0	5677
			32.50	86	0	2189
			35.73	99	0	7030
938303	***srfAB***	Q04747	52.12	81	0	4810
			39.30	94	0	3418
			32.82	86	0	2093
			36.08	99	0	6466
938308	***srfAC***	Q08787	51.26	99	0	2900
			36.13	82	0	1599
			33.11	81	0	1270
938300	***srfAD***	Q08788	52.34	96	2e-83	268
8303165	***slrA***	P0C8M5	52.78	69	9e-08	43.1
938581	***slrR***	P71049	65.79	99	1e-62	204
938417	***glnK***	P40758	53.86	100	4e-142	444
938965	***resE***	P35164	72.16	100	0	1435
			30.35	81	2e-59	507
937404	***lytS***	P94513	66.50	98	0	791
936777	***lytC***	Q02114	51.21	100	3e-165	1170
936150	***sigH***	P17869	91.28	100	1e-132	408

***** Gene IDs and protein IDs are derived from the genome of the reference strain *Bacillus subtilis* 168.

Conclusions

The genome analysis of *Bacillus safensis* P1.5S revealed its strong potential to promote plant growth through both direct and indirect mechanisms. Our *in silico* investigation identified gene clusters related to nitrogen fixation and nitrogen metabolism. Particularly noteworthy are the genes associated with the production of two key plant hormones - indole-3-acetic acid (12 genes) and salicylic acid (7 genes) and the biosynthesis of two volatile organic compounds (acetoin and 2,3-butanediol), molecules which play significant roles in promoting plant growth. Furthermore, the genome harbors 12 biosynthetic gene clusters responsible for secondary metabolites, including lipopeptides, peptides and polyketides which exhibit antifungal, antibacterial, antibiofilm and biosurfactant properties. In addition, the presence of genes involved in the production of siderophores, hydrolytic enzymes or VOCs highlights the P1.5S strain promising biocontrol potential. Genes linked to plant root colonization - such as those involved in chemotaxis, motility, *quorum sensing*, exopolysaccharide production, and biofilm formation - further support the strain plant promoting potential. Taken together, these genomic features underscore the potential of *Bacillus safensis* P1.5S as a promising plant-beneficial

microorganism for sustainable agricultural applications. However, further greenhouse and field studies are essential to validate its efficacy under real-world conditions.

Funding information for the work
This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest
No conflict of interest declared.

Data availability
The data underlying this article are available in GenBank under the accession JARZFW000000000, BioProject number PRJNA960951, BioSample number SAMN34340091, and Sequence Read Archive accession number SRX20079035.

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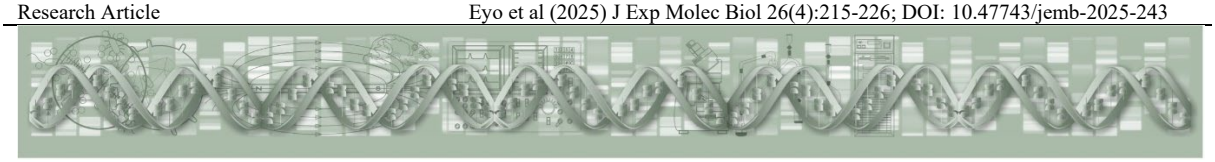

HAEMATOLOGY, BIOCHEMICAL RESPONSES, AND LIPID PROFILES IN DOES SUPPLEMENTED WITH TURMERIC RHIZOME (*CURCUMA LONGA)*

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Abstract

The use of natural feed additives in rabbit diets has gained prominence, particularly those that can enhance growth performance, immunity, and health, while mitigating environmental risks. This study was undertaken to examine the haematological characteristics, serum biochemical indices, and lipid profiles of rabbit does following dietary supplementation with turmeric (*Curcuma longa*) rhizome meal. A total of twenty-four rabbit does were randomly distributed into four treatment groups comprising six animals each, with each group further divided into three replicates of two does per unit. The animals were raised under intensive management in wired hutches, with all necessary welfare protocols observed. Four experimental diets were prepared to include turmeric rhizome meal at 0.0%, 1.0%, 2.0%, and 3.0% inclusion levels. At the end of a 16-week feeding period, 3 mL of blood was collected from a randomly selected doe per replicate into EDTA-treated tubes for haematological analysis. Additional samples were collected for serum biochemical assays. Data were analyzed using analysis of variance (ANOVA) under a completely randomized design (CRD) with SPSS version 21. The results indicated that turmeric supplementation significantly ($p<0.05$) affected packed cell volume (PCV), red blood cell (RBC) count, white blood cell (WBC) count, haemoglobin concentration (Hb), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC). PCV was highest in groups T3 and T1, with values of 39.33% and 38.00%, respectively. Significant ($p<0.05$) effects were also observed on serum urea, enzymes, and creatinine levels. Total protein values ranged from 64.50 to 71.50 g/dL. These findings suggest that incorporating turmeric at a 2.0% dietary level enhances haematological and biochemical profiles, thereby promoting improved health status in rabbit does with positive health benefits on human health.

Keywords: haematology, biochemical responses lipid profile, turmeric, does, *Curcuma longa*, rhizome

Introduction

Commercial rabbit farming has experienced a resurgence owing to the species' high reproductive rate, rapid growth, and lean meat yield (Savietto et al. 2015, Mínguez 2014, Ricke et al. 2012). Rabbits require relatively inexpensive feed inputs and housing, and they exhibit lower competition with humans for cereal grains compared to poultry and pigs (Petrescu et al. 2013). Their meat is nutritionally rich, containing approximately 22% protein, and is low in fat (4%) and cholesterol (5%), rendering it a healthy dietary option (Nistor et al. 2013). Nistor et al. (2013) also reported that rabbit meat has superior calcium (21.4 mg/100 g) and phosphorus

Received 27 May 2025; Revised 02 December 2025; Accepted 5 December 2025;
Published Online 8 December 2025; Published 23 December 2025

(347 mg/100 g) content, with relatively reduced levels of fat (9.2 g/100 g) and cholesterol (56.4 mg/100 g) when compared to other meat sources.

The use of natural feed additives in rabbit diets has gained prominence, particularly those that can enhance growth performance, immunity, and health, while mitigating environmental risks (Foldesiova et al. 2015). Turmeric (*Curcuma longa*) has emerged as a promising phytogenic feed additive, renowned for its anti-inflammatory, antioxidant, and antimicrobial properties, attributed largely to curcuminoids and essential oils (Nelson et al. 2017). Turmeric rhizome consists of 60–70% carbohydrates, 6–13% moisture, 6–8% protein, 5–10% fat, and 3–7% each of dietary minerals, essential oils, and fiber (Nelson et al. 2017). It is rich in turmerone, germacrone, atlantone, zingiberene, and micronutrients such as riboflavin, niacin, thiamine, calcium, potassium, phosphorus, and iron (Hong et al. 2014, Hu et al. 2014, Nwankwo 2014).

Haematological assessment serves as a reliable tool for evaluating the physiological and health status of livestock. It plays an integral role in diagnosing anaemia, infection, and immune responses, and in monitoring the influence of dietary supplements (Maxwell 2013, Rasko 2013, Etim et al. 2014). Parameters such as RBC, WBC, Hb, and PCV are pivotal indicators of health and can reflect genetic resilience to environmental stressors and disease (Isaac et al. 2013).Therefore, this study was designed to investigate the effects of dietary inclusion of turmeric rhizome meal on the haematological indices, serum biochemistry, and lipid profile of rabbit does, with a view to assessing its potential as a functional feed additive for enhancing animal health and productivity.

Materials and Methods

Study Site/ Location

The research was conducted at the Rabbitry Unit of the Teaching and Research Farm, Department of Animal Science, University of Uyo, Akwa Ibom State, Nigeria. The study area is situated at a latitude of 5.0377°N and a longitude of 7.9128°E, with an elevation above sea level. The region experiences an average annual rainfall of approximately 2500 mm and a mean annual temperature ranging from 27 °C to 28 °C (World Weather Online, 2021).

Procurement and Processing of Turmeric Powder Meal

Fresh turmeric rhizomes (*Curcuma longa*) were procured from the Akpan Andem Market, Uyo Local Government Area. The rhizomes were thoroughly washed, sliced into manageable portions, and subsequently sun-dried until fully dried. The dried rhizomes were then milled into fine powder using an electric grinding machine. The turmeric powder was stored in airtight, dry containers to preserve quality and prevent contamination or fungal infestation prior to incorporation into the experimental diets.

Experimental Diets

Four dietary treatments were formulated to meet the nutritional requirements of rabbit does as recommended by the National Research Council (NRC, 1994). These diets were supplemented with turmeric powder at inclusion rates of 0.0%, 1.0%, 2.0%, and 3.0%, and designated as T1, T2, T3, and T4 respectively.

Experimental Design and Protocol

A total of twenty-four (24) mongrel rabbit does were used for the study, structured in a Completely Randomized Design (CRD). The does were randomly allotted into four treatment groups, each containing six animals, further subdivided into three replicates of two does per replicate. The animals were housed in individual wire-mesh cages and provided with consistent care under intensive management conditions.

Table 1. Composition of Experimental Diets to be fed to Experimental Animals

Ingredients (%)	**T_1 (0.0%TPM)**	**T_2 (1.0% TPM)**	**T_3 (2.0% TPM)**	**T_4 (3.0% TPM)**
Maize	35.00	35.00	35.00	35.00
Soybean	15.00	15.00	15.00	15.00
Wheat offal	46.00	46.00	46.00	46.00
Bone meal	3.00	3.00	3.00	3.00
Common salt	0.25	0.25	0.25	0.25
Premix*	0.25	0.25	0.25	0.25
Lysine	0.25	0.25	0.25	0.25
Methionine	0.25	0.25	0.25	0.25
Total	**100.00**	**100.00**	**100.00**	**100.00**
Calculated Nutrient Content (%)				
Metabolized Energy (Kcal/Kg)	2824.10	2833.9	2866.9	2899.8
Crude Protein (%)	17.72	17.72	17.73	17.73
Crude Fibre (%)	5.59	5.53	5.55	5.58
Ether Extract (%)	3.61	4.23	4.88	5.53

*To provide the following per kilogram of diet: Vitamin premix = Vitamin A (12,000,000 IU); Vitamin D_3 (2,500,000IU); Vitamin E (30,000 IU); Vitamin K (2,500mg); Vitamin B1 (2,250mg); Vitamin B2 (6,000mg); Vitamin B6 (4,500mg); Vitamin B12 (15mg); Niacin (40,000mg) ; Pantothenic Acid (15,000mg); Folic Acid (1,500mg); Biotin (50mg); Choline chloride (300,000mg); Manganese (80,000mg); Zinc (50,000mg); Iron (20,000mg); Copper (5,000mg); Iodine (1,000mg); Selenium (200mg); Cobalt (500mg); and Antioxidants (125,500mg).

Data collection

Haematological Profile Examination

At the end of the 14-week feeding period, blood samples (3 mL) were aseptically collected from the ear vein of one randomly selected doe per replicate using a sterile 5 mL disposable syringe with a 22-gauge needle between 7:00 and 8:00 AM. Samples were transferred into EDTA-treated tubes for haematological analysis. Parameters such as packed cell volume (PCV), red blood cells (RBC), white blood cells (WBC), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were analyzed using an automated haematology analyzer.

Serum Biochemical Profile Determination

Another 3 mL of blood was drawn from the ear vein of each replicate animal into plain sample bottles without anticoagulant. The blood was allowed to clot and the serum was separated by centrifugation. Biochemical parameters analyzed included total protein (albumin and globulin), urea, glucose, alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST). Additionally, ALT, AST, and ALP levels were evaluated using an Audiocomb Serum Auto-analyzer (Bayer Express Plus, Bayer Germany, Serial No. 15950).

Lipid Profile

For lipid profile analysis, 3 mL of blood was collected from the ear vein into plain tubes. The sera were separated via centrifugation at 4000 rpm for 5 minutes at 20°C. The samples were analyzed at the University of Uyo Teaching Hospital using a biochemical autoanalyzer (Cobas Mira Plus, Roche Diagnostics) to determine concentrations of triglycerides, total cholesterol, high-density lipoprotein (HDL), and low-density lipoprotein (LDL).

Statistical analysis

All data collected were subjected to analysis of variance (ANOVA) using the IBM SPSS Statistics software version 21, following a completely randomized design. Treatment means were separated using Duncan's Multiple Range Test as described by Duncan (1955).

Statistical model used:

$$X_{ij} = \mu + Y_i + \Sigma_{ij}$$

Where: X_{ij} = Individual Observation (haematological indices, lipid profile and serum biochemical)

μ = Overall Mean

Y_i = Effect of Treatment (turmeric powder)

Σ_{ij} = Experimental Error

Results

Haematological profile of rabbit does fed diets supplemented with turmeric rhizome powder

The effects of dietary supplementation of turmeric powder (TP) on haematological parameters in rabbit does are summarized in Table 2. Significant differences ($p<0.05$) were observed across treatments for packed cell volume (PCV), white blood cell (WBC) count, red blood cell (RBC) count, haemoglobin (Hb), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC). The highest PCV values were recorded in does fed the T3 (2.0% TP) and control (T1) diets, with values of 39.33% and 38.00%, respectively, while the lowest value was observed in T4 (3.0% TP) at 34.00%.White blood cell count was significantly elevated ($p<0.05$) in T2 does (9.45×10^9/dL), compared to those in T1, T3, and T4, which exhibited statistically similar but lower values. Platelet counts ranged from 116.00 to 181.50 $\times10^9$/dL across the treatments and showed no significant differences. RBC values ranged from 5.05 to 5.97×10^{12}/L, with T3 does recording the highest count. Although RBC values in T1, T2, and T3 were statistically similar ($p>0.05$), does in T4 recorded significantly lower values. No significant variation was noted in mean corpuscular volume (MCV) among treatments. However, significant differences ($p<0.05$) were found in MCH and MCHC, with the highest MCHC recorded in T4 (32.50%). Haemoglobin concentration was significantly higher ($p<0.05$) in T3 (12.30 g/dL), followed by T1 (11.90 g/dL), T2 (11.45 g/dL), and T4 (11.25 g/dL). Lymphocyte and neutrophil percentages were unaffected ($p>0.05$) by turmeric inclusion. Lymphocyte counts increased progressively from T1 to T4 (52.00% to 60.00%), while neutrophils decreased (42.50% to 34.00%), but these trends were not statistically significant.

Table 2. Haematological profile of rabbit does fed diets supplemented with turmeric powder

Parameters	T1 (0.0% TP)	T2 (1.0% TP)	T3 (2.0% TP)	T4 (3.0% TP)	SEM
Packed Cell Volume (%)	38.00^{ab}	36.00^{bc}	39.33^{a}	34.50^{c}	0.65
White Blood Cells ($\times10^9$/dL)	7.25^{b}	9.45^{a}	6.37^{b}	5.30^{b}	0.54
Platelet	181.50^{a}	116.00^{c}	151.00^{b}	133.00^{bc}	13.07
Red Blood Cells ($\times10^{12}$/L)	5.70^{ab}	5.75^{ab}	5.97^{a}	5.05^{b}	0.14
MCV (fl)	66.65	63.00	66.33	69.00	1.01
MCH (pg)	21.00^{ab}	20.50^{b}	21.00^{ab}	22.50^{a}	0.32
MCHC (%)	31.50^{bc}	32.00^{ab}	31.00^{c}	32.50^{a}	0.19
Haemoglobin (g/dL)	11.90^{ab}	11.45^{ab}	12.30^{a}	11.25^{b}	0.17

Lymphocytes (%)	52.00	54.50	56.33	60.00	2.67
Neutrophils (%)	42.50	40.00	38.00	34.00	2.58

MCV - mean corpuscular volume, MCH – mean corpuscular haemoglobin, MCHC – Mean corpuscular haemoglobin concentration, SEM – Standard error of means; Means with different superscripts are significant ($p<0.05$)

Effect of turmeric supplementation on serum biochemical indices in rabbit does

The results on serum biochemistry in rabbit does fed diets supplemented with turmeric meal are presented in Table 3. The result revealed that turmeric significantly influenced ($p<0.05$) blood urea level, serum enzymes and creatinine. The mean total proteins in does fed diets supplemented with turmeric meal were 66.00, 64.50, 71.50 and 69.50 g/dL. Turmeric supplementation significantly affected ($p<0.05$) urea in does with higher mean values in does fed T3 (6.80 mmol/L), which was similar ($p>0.05$) to does in T2 (6.00 mmol/L), but different ($p<0.05$) for does in T1 (5.70 mmol/L) and T4 (5.60 mmol/L) respectively.

Serum enzymes were all influenced ($p<0.05$) by dietary supplementation of turmeric meal in the rabbit does' diets in the current study. Aspartate aminotransferase (AST) was elevated ($p<0.05$) in does fed diets supplemented with turmeric in T4 (39.00 µ/L) and T3 (34.00 µ/L). The mean AST values of does observed in this study were similar ($p>0.05$) in T1 (23.00 µ/L) and T2 (23.00 µ/L) which were significantly lower ($p<0.05$) that those of does in T4 and T3 respectively. Higher values ($p<0.05$) of mean alanine aminotransferase (ALT) was observed in rabbit does fed diets supplemented with turmeric meal in treatment groups 4 and 3 with mean values of 38.00 and 30.00 µ/L respectively while does fed T1 and T2 shared similar ($p>0.05$) lower values of 22.00 and 23.00 µ/L respectively. Turmeric meal supplementation increased ($p<0.05$) alkaline aminotransferase (ALT) in rabbit does in this study, although does in T2 had similar statistical value with those in the control group (T1), without turmeric supplementation. The meal ALT values recorded were 14.00, 16.50, 21.00 and 23.50 µ/L for T1, T2, T3 and T4 respectively. The values were observed to increase with corresponding higher dose of supplement in the does' diets.

Creatinine values were higher in does with turmeric meal in their diets however, these values were statistically similar in rabbit does in T1, T2 and T4 with mean values of 97.00, 114.00 and 123.00 µ/L respectively. Turmeric meal did not have any significant effect ($p>0.05$) on blood glucose level in the does in this study, although higher non-significant ($p>0.05$) values were seen in rabbit does with turmeric meal in their diets when compared with those of the control group without turmeric meal supplementation in their diets. Glucose values were 4.05, 4.85, 4.95 and 5.45 g/dL for T1, T2, T3 and T4 respectively.

Table 3. Effect of turmeric supplementation on serum biochemical indices in rabbit does

Parameters	**T1 (0.0% TP)**	**T2 (1.0% TP)**	**T3 (2.0% TP)**	**T4 (3.0% TP)**	**SEM**
Total protein (g/dL)	66.00	64.50	71.50	69.50	1.78
Urea (mmol/L)	5.70^{b}	6.00^{ab}	6.80^{a}	5.60^{b}	0.19
AST (µ/L)	23.00^{b}	23.00^{b}	34.00^{a}	39.00^{a}	2.45
ALP (µ/L)	22.00^{b}	23.00^{b}	30.00^{ab}	38.00^{a}	2.25
ALT (µ/L)	14.00^{c}	16.50^{bc}	21.00^{ab}	23.50^{a}	1.32
Creatinine (mg/dL)	97.00^{b}	114.00^{ab}	142.00^{a}	123.00^{ab}	6.52
Glucose (g/dL)	4.05^{c}	4.85^{b}	4.95^{b}	5.45^{a}	0.22

AST - Aspartate aminotransferase; ALP – Alanine amino phosphatase; ALT - Alanine aminotransferase; SEM – Standard error of means; Means with different super Scripts are significant ($p<0.05$).

The effects of turmeric supplementation on lipid profile are displayed in Table 4. Triglyceride levels showed a significant increase in T3 (4.10 mmol/L), while T1, T2, and T4 exhibited

similar and lower values (1.80–2.00 mmol/L). Total cholesterol levels were highest in T3 (4.24 mmol/L) and significantly lower in T1 (2.09 mmol/L). High-density lipoprotein (HDL) levels were significantly elevated in T3 (1.10 mmol/L) and T4 (1.23 mmol/L) compared to T1 and T2 (both at 0.60 mmol/L). Very-low-density lipoprotein (VLDL) concentrations ranged from 0.19 mmol/L in T1 to 0.41 mmol/L in T3, showing significant differences ($p<0.05$). Low-density lipoprotein (LDL) value was highest in T3 (2.60 mmol/L), with the lowest observed in T4 (0.70 mmol/L).

Table 4. Lipid profile of rabbit does fed dietary supplementation of turmeric meal

Parameters	T1 (0.0% TP)	T2 (1.0% TP)	T3 (2.0% TP)	T4 (3.0% TP)	SEM
Triglyceride	2.00^{b}	1.80^{b}	4.10^{a}	1.90^{b}	0.32
Total cholesterol	2.09^{b}	1.77^{c}	4.24^{a}	2.12^{b}	0.14
HDL	0.60^{b}	0.60^{b}	1.10^{a}	1.23^{a}	0.14
VLDL	0.19^{d}	0.37^{b}	0.41^{a}	0.32^{c}	0.03
LDL	1.30^{b}	0.80^{c}	2.60^{a}	0.70^{c}	0.23

Discussions

Haematolocal profile of rabbit does fed diets supplemented with turmeric rhizome powder

According to Tobou et al. (2020), blood parameters are considered as the main pathological, nutritional and physiological indices for assessing the state of an organism. Therefore, any change in the constituent elements of blood relative to the normal values is considered an important index for the interpretation of the physiological or metabolic state of the animal, especially the quality of feed.

Turmeric powder supplementation significantly affected the PCV, WBC, RBC, Hb, MCH and MCHC respectively in rabbit does in this study. This result is consistent with the report of Ayodele et al. (2021) who observed a significantly ($p<0.05$) higher Hb and RBC in pullet chicks fed diets containing turmeric powder when compared with those of the other diet groups. In their study, pullet chicks fed diet supplemented with 1%. Turmeric diet had a significantly higher PCV value than those of the other treatment groups. The highest value of PCV was observed in does fed T3 (1.0% TP). This result however, differs from the report of Daramola et al. (2020) who documented no significant effect of turmeric powder supplementation in PCV, Hb, MCH and MCHC in broiler chicken at up to 1.0% supplementation in their diets. The results on PCV, RBC and Hb agree with those of Adegoke et al. (2018) who also observed significant effect on turmeric in broiler chicken but disagrees with the report of Tobou et al. (2020). The increase in PCV, RBC and Hb at 1.0% supplementation in this study showed that the does did not suffer anemia in the course of the study, since RBCs and PCV are significant in the diagnosis of anaemia (Adegoke et al. 2018), neither was the oxygen - carrying capacity in the does' blood compromised. Okpuzor et al. (2009) in their study noted that an increase in the count of RBC, Hb and PCV is suggestive of polycythemia and positive erythropoiesis. El-Rawi et al. (2020) also had variable influence on blood parameters of guinea pigs with the inclusion of *C. longa* powder in their diets. This suggests that turmeric at 1% inclusion will improve oxygen - carrying capacity of the cells as reported by Isaac et al. (2013) which signifies sufficient iron in the blood.

There was a significant increase in WBC of the does at 0.5% supplemental level compared to those with 0.0, 1.0, and 1.5% supplementations, respectively. This observation is in tandem with previous studies (Adegoke et al. 2018, Tobou et al. 2020), but varies with the results of Ayodele et al. (2021) and Daramola et al. (2020) who did not observe any significant difference

with turmeric powder supplementation on WBC of pullet chicks and broiler chicken, respectively. According to Odesanmi et al. (2010), a decrease in the count of WBC indicates suppression of leucocytes and their production from bone marrow which suggests presence of infection or a regenerative anaemia. Tobou et al. (2020) reported that the incorporation of 0.25% of *C. longa* powder significantly increased white blood cell, hematocrit, lymphocyte, monocyte, granulocyte and platelet concentration in their study. Syed et al. (2018) earlier stated that phytobiotics are also used as blood and immunity purifiers because they stimulate white blood cells and eventually increased interferon levels. Hence, turmeric powder supplementation significantly improved the immunity of the does, thereby increasing their ability to resist or withstand infections in the blood. The supplementation of turmeric powder reduced the platelet values in the does when compared to their counterparts in the control group without turmeric powder supplementation in their diet.

The observed differences in MCH and MCHC are similar to the report of Daramola et al. (2020). They opined that the increase in these parameters is an indication of improving oxygen - carrying capacity of the cells. Haemoglobin, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration are important blood parameters whose values are used to determine the presence and severity of anaemia (Aikpitanyi and Egweh 2020). Tobou et al. (2020) reported a significant difference in guinea pigs with turmeric powder supplementation. This study did not show any significant variation in the lymphocytes and neutrophils of does with supplemental levels of turmeric powder in their diets. When neutrophil number in the blood reaches a critically low level, animals are highly susceptible to bacterial infection (Weiss et al. 2010). Adeyemi (2014) reported that increased numbers of lymphocytes are seen in most viral infections, some bacterial infections, and some cancers while decreased numbers of lymphocytes are seen in steroid exposure, some cancers, immunodeficiency and renal failure. Again, he noted that neutrophils are the most common of the WBCs and serve as the primary defense against infection. According to Adeyemi (2014), the typical response to infection or serious injury is an increased production of neutrophil, and further added that immature forms of neutrophils are seen early in the response to infection. The non-significant effect of turmeric powder supplementation on these parameters suggests the absence of infection in the does in the course of the study.

Serum biochemical indices in rabbit does fed diets supplemented with turmeric powder.

Although turmeric powder supplementation did not show a statistically important effect on total protein and blood glucose in this study, urea, creatinine, AST, ALP and ALT on the other hand, were significantly affected. The non-significant effect of turmeric powder supplementation on serum total protein suggest that the supplement did not have a negative effective on protein digestibility, since Ahamefule et al. (2008) attributed high protein in serum to an indication of protein adequacy. The result on total protein in this study is similar to the findings of Tobou et al. (2020), El-Rawi et al. (2020) and Ayodele et al. (2021) who reported non-significant effect of turmeric powder supplementation in their respective studies. Adegoke et al. (2020) however, observed significant variation in their study. Turmeric powder supplementation increased blood urea at 1.0% (T3), which was similar to values of does in T2 but differ from those of does in T1 and T4, which were also similar. The result disagrees with the report of Ayodele et al. (2021) when they studied the effect of turmeric powder supplementation at 0.0, 1.0 and 2% in broiler chicken. This observed difference may be due to specie difference, though both animals are monogastric. This significant increase in serum urea concentration could be attributed to ammonia production dies in the caecum that is similar to that of the rumen in ruminants as noted by Abdel El-Latif et al. (2019). Serum urea concentration is an index that reflects the state of protein metabolism, renal function and body nutrition as noted by Kryshtafovych et al. (2014). Uric-acid in the blood, as described by Chernecky and Berger (2008), is produced as a

result of protein metabolism. Hence, increased protein metabolism as well as stress and dehydration can affect the concentration of Uric acid in the blood.

There was significant difference in AST of rabbit does at 1.0% (T3) and 1.5% (T4), turmeric meal supplementation in this study. These values were higher than those of does in 0.0% (T1) and 0.5 % (T2) respectively. Similar observation was made for ALP. The ALT values in the does were also elevated with turmeric powder supplementation although the value for does in T2 (0.5%), was similar to those of does in the control group. The liver as stated by Aikpitanyi and Egweh (2020) is the center of several digestive, metabolic and productive activities, and as such, is susceptible to a varying degree of chemical and biological damages. These damages, they said, are made obvious by the serum levels of specific enzymes originating from the liver, which depending on their levels may cause some disruptions to bodily functions, thereby resulting in poor health and production performance (Aikpitanyi and Egweh 2020). Aikpitanyi and Egweh (2020) described the activities of AST, ALP and ALT in the blood as bioindicators of liver function and damage. Lumeij (2008) also noted that increased levels of these enzymes are associated with liver or muscle damage, resulting from the body's response to stress. This therefore, suggests that the does with 1.0 and 1.5% supplementation may have suffered some levels of liver damage in the course of the study. According to Lumeij (2008), higher serum levels of ALP are observed when there is increased osteoblastic activity, involving the formation and mineralization of bone associated with increased skeletal growth. Tobou et al. 2020) also observed increased serum urea, AST and ALT levels with the inclusion of turmeric powder in the rations.

Creatinine is used to determine the status of the kidney (Ayodele et al. 2021). The primary function of the kidney is to excrete waste products resulting from protein metabolism and muscle contraction as stated by Ileke et al. (2014). Creatinine values increased with turmeric powder supplementation, although does without turmeric powder supplementation and does with 0.5% and 1.5% respectively, had similar creatinine values. Esuboteng in Ayodele et al. (2021) opined that creatinine is excreted by the kidney as a by-product of creatinine phosphate metabolism which is produced as a result of energy production by the skeletal muscles. The elevated value in does fed diets containing 1.0% turmeric powder as a supplement suggests that they may have been exposed to kidney damage; hence, the elevated value in the blood. Turmeric powder supplementation did not influence glucose values in this study.

Lipid profile of rabbit does fed dietary supplementation of turmeric meal

The result on triglycerides showed significant differences, with higher value been observed in does fed the T3 diet. This result differs from the report of El-Rawi et al. (2020) who observed significant reduction in triglyderides in rabbits fed turmeric in their diets. The rabbit does in T3 exhibited a higher total cholesterol value than those of does in T1, T2, and T4 respectively. Nwoko et al. (2022) reported that TC, TG, LDL and VLDL levels were significantly lower for rabbits in T4 compared to treatments 1, 2 and 3 and added that these parameters decreased as Turmeric increased in the diet which contradicts the current study. Dehzada et al. (2023) in their abstract concluded that turmeric/curcumin supplementation seems to be effective in improving blood levels of TC, TG, LDL and HDL. The significant increase in TC and V-LDL in this study disagrees with the findings of Nwoko et al. (2022) who found decrease in TC, LDL, VLDL and suggested that turmeric rhizome powder is a hypocholesterolemic additive. El-Rawi et al. (2020) attributed the decrease in total lipid and cholesterol in their work to the effect of essential oil compounds present in the turmeric on lipid metabolism or due to curcumin that enhances bile production and hence, lipid digestion. The authors also reported that liver triacylglycerol and cholesterol concentrations were considerably less in rats fed curcumin than in control animals. Results on LDL showed significantly higher value at T3 but were significantly lower in rabbit does fed T2 and T4 diets respectively. LDL is known as bad cholesterol because it carries fat from the liver to the blood vessels and encourages arterial

cholesterol deposition (Nwoko et al. 2022). Earlier reports (Benzie and Galor 2011, Woode et al. 2011) suggested serum lipids lowering properties of turmeric rhizome powder.

Conclusions

From the findings of this study, it can be concluded that supplementing rabbit does' diets with 2.0% turmeric rhizome meal can improve blood parameters and enhance the overall health of the animals.

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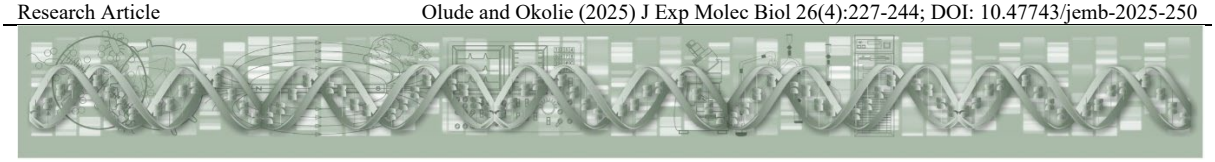

FICUS EXASPERATA SUPPRESSES EARLY EVENTS IN COLORECTAL CARCINOGENESIS BY DOWNREGULATING THE EXPRESSION OF BETA-CATENIN AND ENHANCING ADENOMATOUS POLYPOSIS COLI GENE EXPRESSION

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Abstract

Wingless (Wnt) signalling is an important evolutionarily conserved signalling pathway in animals that regulates biological processes such as development, cell growth, and differentiation. Abnormalities in the components of the Wnt/β-catenin signalling pathway have been a major cause of cancer, especially in colorectal cancer (CRC). This study investigates the effect of the methanol leaf extract of *Ficus exasperata* (MEFE) on the wnt signalling pathway, considering beta-catenin and adenomatous polyposis coli (APC) as key markers. This study further delves into the quantitative and qualitative phytochemicals present in the leaf extract. Malondialdehyde (MDA), reduced glutathione (GSH) level and some haematological indices were also assayed for, in the test animals. A total of forty-eight Wistar rats, grouped into 8 cages, were used for this study. The control group was the first group; group 2 was treated with extract alone (500 mg/kg body weight); group 3 rats were injected subcutaneously with 40 mg/kg b.w. of 1,2- dimethylhydrazine (DMH) twice a week; group 4 was treated with both the leaf extract (500 mg/kg b.w.) and DMH; group 5 was treated with the leaves extract (750 mg/kg b.w.) and DMH; group 6 was pretreated with the leaf extract (500 mg/kg b.w.) before the administration of DMH; group 7 (post-treated) was given DMH for some weeks before the commencement of treatment with the leaf extract (500 mg/kg b.w.); group 8 was given the carcinogen and treated with a standard drug (12.5 mg/kg b.w. of 5-fluorouracil} simultaneously. Appreciable amount of phenol, flavonoid, tannin and anthocyanidin were present in the plant extract. Alkaloids, terpenoids, phytosterols, saponins and anthraquinones were also found in the plant. The immune system of the rats was strengthened by the extract. The haemoglobin and red blood cell levels of rats treated with the plant extract were within the normal range compared to the control ($p<0.05$). Reduced glutathione level and adenomatous polyposis gene were reduced while, malondialdehyde level and beta-catenin gene expression were statistically significantly increased in carcinogen-only treated groups compared to other groups ($p < 0.05$). The plant was able to inhibit oxidative stress and also suppress the expression of β-catenin while enhancing the expression of adenomatous polyposis coli. These potentials might be a s a result of the phytochemicals present in the plant extract.

Keywords: *Ficus exasperata*, colorectal carcinogenesis, beta-catenin, Adenomatous polyposis coli

Received 19 August 2025; Revised 01 December 2025; Accepted 05 December 2025;
Published Online 8 December 2025; Published 23 December 2025

Introduction

Colorectal cancer (CRC) arises from either the rectum or colon. The epithelial cells of the large intestine are known for the dynamics of consistent cell renewal, which makes it prone to proliferation disorders. Formation of polyps is one of the disadvantages of the high cell renewal dynamics, though it's benign, but under certain circumstances it can transit into the formation of malignant tumours and hence cancer (Kowalska et al. 2025). Initiation to metastasis in CRC is influenced by genetics, epigenetics, molecular alterations, mutation in oncogenes, mutation in tumour suppressor genes, and alterations in signalling pathways that control apoptosis, cell proliferation, cell survival and cell differentiation (Papavassiliou et al. 2024, Delle-cave 2025). Colorectal cancer (CRC) remains a threat to human health and the need to provide a lasting cure to this ailment is crucial. CRC is a solid tumor associated with the disorder of the Wnt/β-catenin signalling pathway. Wnt/βcatenin or the canonical Wnt signalling pathway, the planar cell polarity (Wnt-PCP) pathway and the Wnt-Ca2+ signaling pathway are classes of the Wnt signalling cascade (Nusse and Clevers 2017). The Wnt/β-catenin signalling pathway is associated with physiological processes, but dysregulation of this pathway has been linked to solid tumors and haematological disorders (Clevers and Nusse 2012, Cheng et al. 2019).

Normal physiological environment entails the transcription factor β-catenin, an important molecule in the Wnt/βcatenin or the canonical Wnt signaling pathway, being degraded by a complex called the β-catenin destruction complex. The destruction complex consists mainly of two kinases: glycogen synthase kinase 3β (GSK3β), casein kinase I (CK I) and two scaffolds: adenomatous polyposis (APC) and axis inhibition (Axin) (Kim et al. 2009; Mantilla et al. 2015). Casein kinase I and GSK3β phosphorylate β-catenin. CK 1 phosphorylates β-catenin at serine 45, Ser33, and Ser37 while GSK3β phosphorylates at threonine 41 (Thr41) (Koni et al. 2020). The phosphorylation of β-catenin makes it susceptible and recognised by E3 ubiquitin ligase (β-transducin repeat-containing protein: β-TrCP) for ubiquitination and proteasomal degradation (Zhu and Li 2023). The degradation inhibits the translocation of β-catenin into the nucleus, while allowing histone deacetylation, chromatin compaction and Groucho-mediated promoter repression. Hence, transcription is halted (MacDonald et al. 2009, Jackstadt et al. 2020). Phosphorylation, the frizzled (FZD) family receptors, and the low-density-lipoprotein-related protein 5/6 (LRP5/LRP6) co-receptors are required for the activation of the canonical Wnt signals (Gajos-Michniewicz et al. 2020). The binding of the Wnt ligands to its receptors results in dishevelled phosphorylation and release of β-catenin from the destruction complex. The release prevents β-catenin ubiquitination and degradation. Increase and accumulation of β-catenin aids its nuclear translocation. In the nucleus, the Groucho repressor undergoes displacement, allowing β-catenin to interact with the T-cell factor/lymphoid enhancer factor (TCF/LEF), chromatin remodelling and transcription of genes such as c-myc and cyclin D1 (Zhan et al. 2017). Anastas and Moon (2013), in their review, reported that the role of the Wnt in mouse models of mammary cancer and in human and mouse colon cancer was first described over 30 years ago. Nusse et al. (1982) and Nusse et al. (1984) also reported spontaneous mammary hyperplasia and tumours in mice induced by a proviral insertion at the Wnt1 locus, leading to aberrant overexpression of WNT1. Wnt1 transgenic mice similarly develop mammary tumours, suggesting a causative role for WNT1 in mammary tumorigenesis (Tsukamoto et al. 1988). Koni et al. (2020) reported that Wnt/β-catenin signalling pathways play a crucial role in carcinogenesis of all ovarian cancer subtypes (Jeong et al. 2009), glioma (Zhao et al. 2020), prostate cancer (Situ et al. 2020), osteosarcoma (Nomura et al. 2019), melanoma (Muralidhar et al. 2019), and pancreatic cancer (Chen et al. 2020). Hyperactive Wnt/β-catenin and elevated intracellular β-catenin have also been implicated in breast tumours (Khramtsov et al. 2010, Sormunen et al. 1999). Over 90% of non-metastasising fibromatosis

and metaplastic carcinomas have been associated with the high level of β-catenin expression (Lacroix-Triki et al. 2010).

A major hallmark of colorectal cancer is the hyperactivation of this canonical Wnt signalling associated with a mutation in the tumour suppressor gene, APC (Tewari et al. 2021). Loss of a functional adenomatous polyposis coli (APC) is one of the initiating factors of colorectal cancer (Noe et al. 2021). The APC gene, located on chromosome 5q regulates the β-catenin /WNT pathway, which in turn facilitates differentiation and growth of cells (Rubinfeld et al. 1993). Mutation of the APC gene inhibits the formation of the complex necessary for β-catenin degradation (Sakanaka et al. 1998). The absence of this destruction complex enables and promotes β-catenin accumulation, leading to gastrointestinal epithelial stem cell carcinogenic proliferation (Noe et al. 2021). This signalling cascade remains crucial and important in many biological physiological processes, including embryonic development, cell cycle regulation, apoptosis, inflammation, and cancer (Tai et al. 2015). This cascade determines cell survival, cell fate, cell differentiation, and survival (Yamamoto et al. 2022). The Wnt pathway components have been identified as reliable biomarkers and potential targets for cancer treatment (Zhao et al. 2022).

This study aims to investigate the effect of *Ficus exasperata* on beta-catenin and adenomatous polyposis coli expression in colorectal carcinogenesis. The phytochemicals present in the plants were also assayed, as well as the effect of the plant on some haematological parameters and oxidative stress in CRC.

Materials and Methods

Extraction of Plant

In a local farm situated in Benin City, Edo State, Nigeria, *Ficus exasperata* leaves were harvested and taken to the Department of Plant Biology and Biotechnology in the University of Benin for identification by Prof Henry Akinnibosun. The leaves were properly air dried for three weeks, they were ground, weighed, and macerated for 72hours in methanol. This was stirred at intervals. After 72 hours, it was decanted and freeze-dried using a freeze-dryer. The result was a powdered extract kept in a container and preserved at 4 ºC.

Chemicals and reagents

1,2-Dimethylhydrazine (DMH) was obtained from Sigma Aldrich, Germany. All reagents used were of analytical grade and had the highest purity.

Preparation of the Carcinogen Used

Colorectal cancer was induced in experimental rats using a potent carcinogen named 1,2-dimethyl hydrazine (DMH). DMH was administered subcutaneously (40 mg/kg body weight) two times in a week. It was prepared according to the method of Chari et al. (2018).

Animal grouping and Sacrifice

The experiment lasted for 12 weeks. Wistar rats weighing more than 150 g were used in this study. A total of 8 groups comprising 6 animals each were allowed to acclimatized for two weeks. The first group was the control group, the second group was the positive control group (500mg/kg body weight of extract only), and the negative control was group 3 (40 mg/kg body weight of DMH only), the fourth and fifth group received DMH and the extract together but at different concentrations (500 and 750 mg /kg body weight of extract, respectively). The sixth group was administered the leaf extract before carcinogen treatment, while the seventh group was treated with carcinogen before administering the extract. The eighth group was concomitantly treated with both the carcinogen and the standard drug (12.5 mg/kg body weight of 5-fluorouracil intraperitoneally). At the end of the stipulated weeks, the animals were fasted overnight and sacrificed. Blood samples were collected, and the colons of the animals were also excised for heamatological and biochemical assays.

Tissue homogenate preparations were done using a weighed portion of the excised organs. The organs (1 g) were homogenised in normal saline solution (10 ml), centrifuged, and the supernatant obtained was labelled according to the groupings. This was used for reduced glutathione (GSH) and lipid peroxidation assay marker (malondialdehyde) assays.

Biochemical Assays

Malondialdehyde (MDA) levels were estimated by the method of Burge and Aust (1978). It is one of the products of lipid peroxidation which forms a pink colour when reacted with 2-thiobarbituric acid. The reaction result is read at 535nm wavelength.

Reduced glutathione was estimated according to the method of Ellman (1959). the reaction between GSH and 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB), also known as Ellman's reagent, produces the TNB chromophore and reads spectrophotometrically at 412 nm.

A Gene expression study of beta-catenin and adenomatous polyposis: This was done according to the method described by Elekofehinti et al. (2020). The Quick-RNA MiniPrep™ Kit (Zymo Research) was used to extract the total RNA from the tissue samples, and DNAse I treatment (NEB, Cat: M0303S), employed for the elimination of DNA contaminants. One microgramme (1 µg) of DNA-free RNA underwent a reverse transcriptase reaction with a cDNA synthesis kit based on ProtoScript II first-strand technology (New England BioLabs). The reaction occurred in three steps: 65 °C for 5 min, 42 °C for 1 h, and 80 °C for 5 min (Elekofehinti et al. 2020). Polymerase chain reaction (PCR) for gene amplification utilized OneTaqR2X Master Mix (NEB) with specific primers (Inqaba Biotec, Hatfield, South Africa). The amplified molecules were resolved on one percent agarose gel (1.0%) followed by the quantification of the gene expressed using the GAPDH gene and "ImageJ" software (Elekofehinti et al. 2020).

Hematological Analysis

The automated haemolyzer was employed to determine the full blood count. Whole blood was used for the determination of hematology. white blood cell, red blood cell, hemoglobin, percentage WBC count (neutrophils, eosinophils and basophils), were analyzed with an automated analyzer (SYSMEX K-21N: SYSMEX CORPORATION, JAPAN).

Phytochemical Screening

The qualitative phytochemical screening followed the methodologies of Harborne (1998), Sofowora (1993), and Trease and Evans (1998), with all samples analysed in triplicate.

Total phenolic compounds: The method of Folin and Ciocalteau (1927) was adopted in the determination of total phenolic content.

Total flavonoid contents: This was determined using the method described by Ayoola et al. (2008).

Proanthocyanidin determination: It was carried out according to the method of Sun et al. (1998).

Data Analysis

Data was statistically analysed using ANOVA, and GraphPad Prism was used in plotting the graphs. Values were presented as mean ± standard error of mean. Data was considered to be statistically significant at $p < 0.05$.

Results

Phytochemical results reveal the presence of tannins, phenols, flavonoids, terpenoids, alkaloids, phytosterols, saponins, anthraquinones and carbohydrates. The spectrophotometric readings reveal that an appreciable amount of total tannins, total flavonoids, proanthocyanidin and total phenolic content was present in the leaf extract of *Ficus exasperata* and shown in Tables 1 and 2.

Table 1. Qualitative phytochemical screening of plant extract

Phytochemicals	
Tannins	Present
Phenol	Present
Flavonoids	Present
Terpenoids	Present
Alkaloids	Present
Reducing sugar	Absent
Phytosterol	Present
Saponins	Present
Catechin	Absent
Anthraquinones	Present
Xanthoproteins	Absent
Carbohydrates	Present

Table 2. Quantitative phytochemical screening of *Ficus exasperata* leaves

Phytochemical	Amount
Total phenolic content (mg GAE/g extract)	25 ± 0.015
Total Flavonoid content (mg QE/g extract)	117 ± 0.029
Proanthocyanidin content (mg AAE/g extract)	235 ± 0.005
Total tannin (mg TAE/g extract)	167.6 ± 0.04

GAE: Gallic acid equivalent; AAE: Ascorbic acid equivalent; QE: Quercetin equivalent; TAE: Tannic acid equivalent. Values are expressed as mean ± SEM, n=3/group

Effect of the Extract on Reduced Glutathione and MDA levels: Figure 1 shows the reduced glutathione (GSH) level of the various groups. The GSH level of groups 3 to 8 was statistically significantly different compared to group 2. The malondialdehyde (MDA) level of the DMH-only group was increased statistically compared to other groups, as displayed in Figure 2.

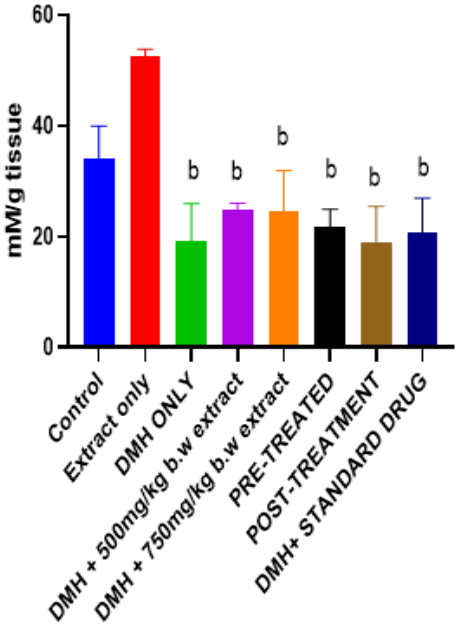

Figure 1. Colon reduced glutathione (GSH) level. Sample size = 6 per group, and values are expressed as mean ± standard error of mean. The alphabet 'b' represents that there was a statistically significant difference ($P < 0.05$) compared to the extract-only group. Reduced

glutathione levels showed no significant difference between the MEFE (cotreated, pretreated and post-treatment) groups and group 8 compared to group 3. All these aforementioned groups differ significantly from group 2. The group that received the carcinogen alone has the lowest level of reduced glutathione.

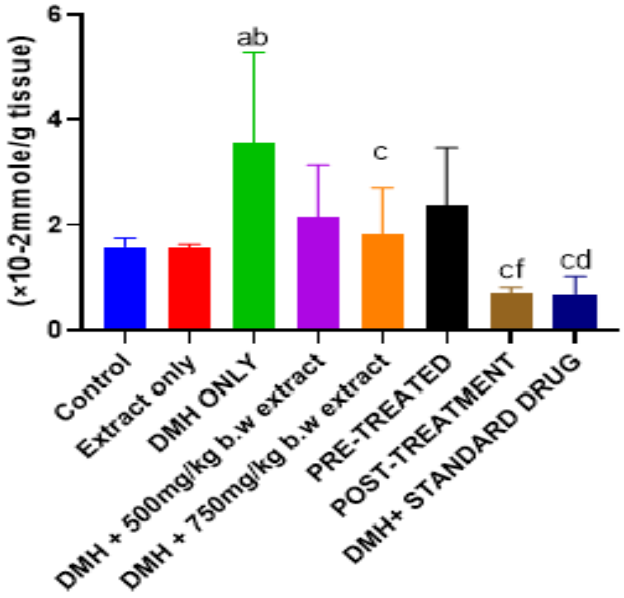

Figure 2. Colon malondialdehyde (MDA) level. Values are expressed as mean ± standard error of mean with a total of six animals in each group. The alphabet represents significant differences at $P < 0.05$. The alphabet 'a' denotes there was a statistically significant difference compared to the control; 'b' denotes there was a statistically significant difference compared to the extract-only group; 'c' denotes there was a statistically significant difference compared to the DMH group; 'd' denotes there was a statistically significant difference compared to group DMH+500 mg/kg b.w.; 'f' denotes there was a statically significant difference compared to the pre-treated group. The group that received DMH only expressed a high level of MDA ($P < 0.05$), which differs from MEFE-treated groups. The MDA level of group 7 that received post-treatment with MEFE was not statistically different from the group treated with standard drugs.

Effect of the Extract on the Expression of beta-Catenin and Adenomatous polyposis coli (APC) gene: Relative expression of the beta-catenin gene displayed in Figure 3 shows that the gene was highly expressed in the DMH-only group. The lowest level of this gene expression was seen in the group treated with the standard drugs, which is closer to the level of the pretreatment group. Adenomatous polyposis relative gene expression is shown in Figure 4. In this result, the pretreated group has the highest relative gene expression of APC, which was statistically different from the remaining groups.

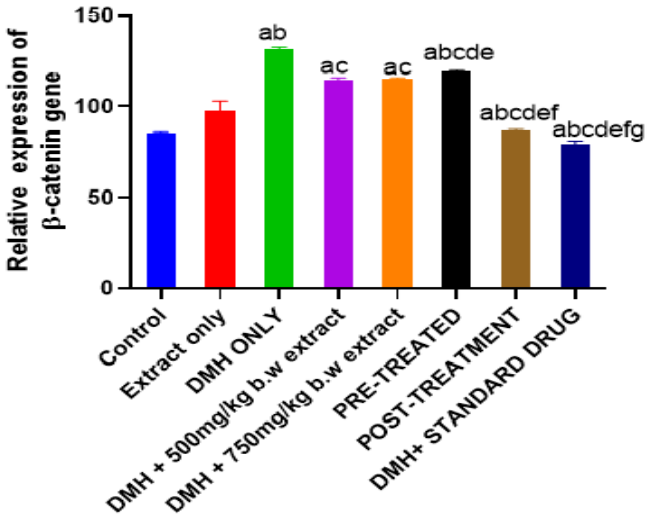

Figure 3. Relative expression of the β-catenin gene. Data are expressed as mean ± standard error with a total of six animals per group. Significant differences ($P < 0.05$) between the groups were represented by lowercase letters. The alphabet 'a' denotes there was a statistically significant difference compared to the control; 'b' denotes there was a statistically significant difference compared to extract-only group; 'c' denotes there was a statistically significant difference compared to the DMH group; 'd' denotes there was a statistically significant difference compared to the group treated with DMH + 500 mg/kg b.w. of extract; f denotes there was a statistically significant difference compared to the pre-treated group, and 'g' denotes there was a statistically significant difference compared to the post-treated group. Each group was statistically different from the others. The post-treated group's beta-catenin gene expression was close to that of groups 1 and 8. There was a high accumulation of beta-catenin in the carcinogen-only treated group.

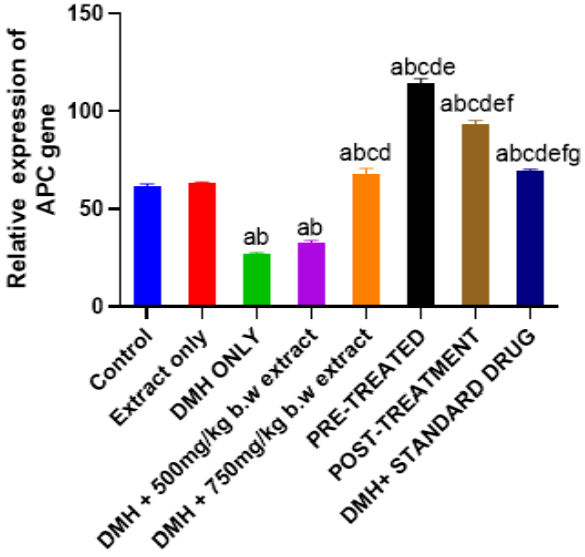

Figure 4. Relative expression of adenomatous polyposis coli (APC) gene. Sample size = 6 per group. Data are expressed as mean ± standard error. Significant differences ($P < 0.05$) between the groups were represented by lowercase letters. 'a' denotes there was a statistically significant difference compared to control; 'b' denotes there was a statistically significant difference compared to extract-only; 'c' denotes there was a statistically significant difference compared to the DMH group; 'd' denotes there was a statistically significant difference compared to the group treated with DMH + 500 mg/kg b.w.; 'f' denotes there was a statistically significant

difference compared to the pre-treated group, and 'g' denotes there was a statistically significant difference compared to the post-treated group. The APC gene was greatly expressed in the pretreated and the post-treated groups compared to other groups.

Effect of the Extract on Haematological Parameters: The haematological result is shown in Figures 5 to 10. The white blood cells, a true picture of the immune system, were statistically reduced in the group treated with the standard drug compared to the pretreatment group ($p<0.05$). Neutrophil levels were high in the DMH-only group, though not statistically significant compared to other groups. The basophil level of DMH+500 mg/kg body weight was reduced compared to other groups, while the group that took the carcinogen with a higher dose of the extract had an increased basophil level that was statistically significant compared to group 4. Red blood cell level showed no statistically significant difference across the groups, though it was a bit reduced in the DMH-only group ($p>0.05$), and the haemoglobin level of group 7 was statistically significantly different from the control ($p<0.05$).

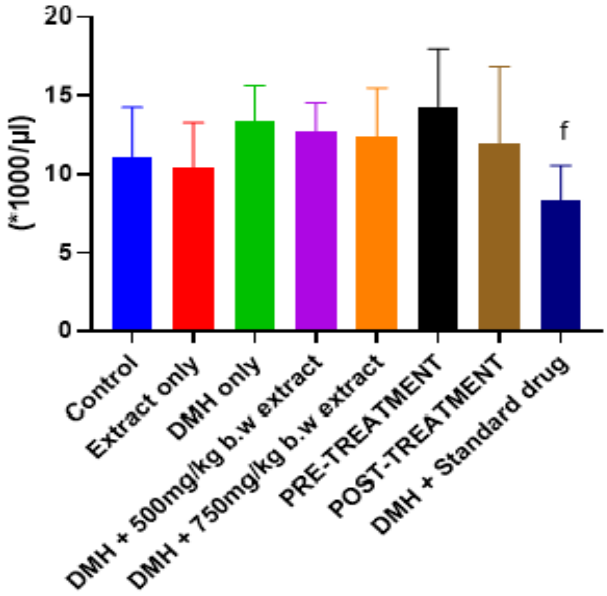

Figure 5. White blood cell count. A sample size of 6 rats per group, and data are expressed as mean ± standard error of means. Lowercase letter represents a significant difference at $P < 0.05$. The alphabet 'f' denotes there was a statistically significant difference compared to the pre-treated group. White blood cell levels showed no significant difference between the groups except for group 8, which differs statistically from group 6.

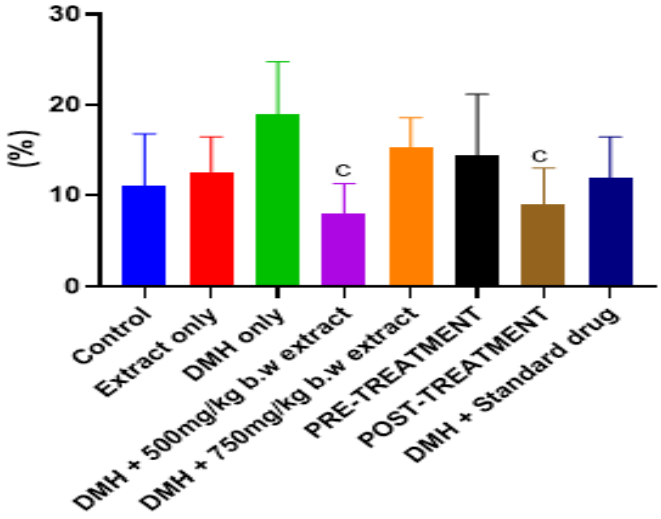

Figure 6. Percentage of neutrophils of rats in different groups. A sample size of 6 rats per group, and data are expressed as mean ± standard error of mean. Lowercase letters 'c' represent a statistically significant difference at $P < 0.05$ compared to the DMH-only group. The neutrophil level of the DMH-only group was greatly increased compared to other groups. Groups 4 and 7 had the lowest level of neutrophil count.

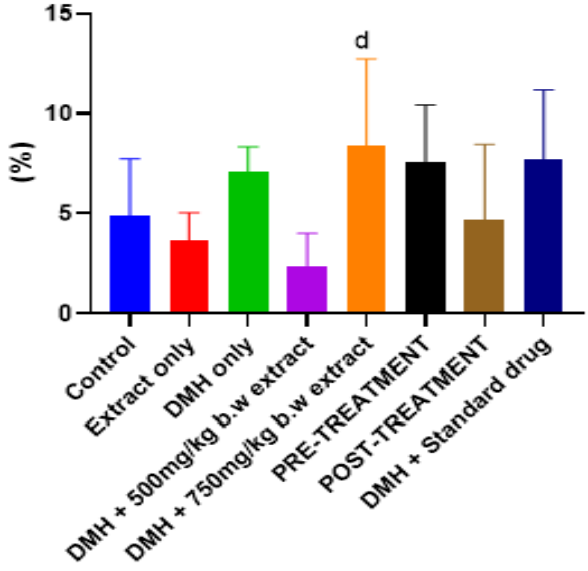

Figure 7. Percentage of basophils in rats of different groups. A sample size of 6 rats per group, and data expressed as mean ± standard error of mean. The alphabet 'd' represents a statistically significant difference at $P < 0.05$ compared to the cotreated group that received DMH + 500 mg/kg b.w. of the extract. The data were statistically insignificant except for group 5, which was significantly higher compared to group 4.

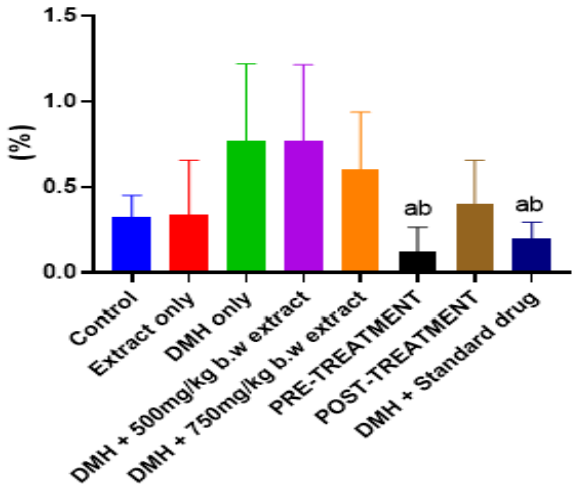

Figure 8. Percentage of eosinophils in rats in different groups. The alphabet 'a' denotes a significant difference from the control, and 'b' denotes a significant difference from the extract-only group. A sample size of 6 rats per group, and data are expressed as mean ± standard error of mean. Groups 1 and 2 were not statistically different from most of the groups except groups 6 and 8. Groups 6 and 8 had the lowest eosinophil count compared to the other groups.

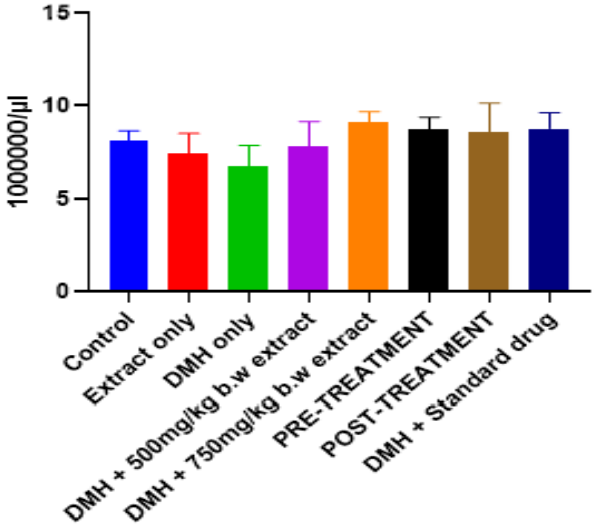

Figure 9. Red blood cell levels of rats in different groups. Values are expressed as mean ± SEM, n=6/group. Values were considered statistically significant at $P < 0.05$. This data also showed no significant difference in red blood cell levels. However, the exposed animals that were treated with MEFE recorded a higher volume of RBC.

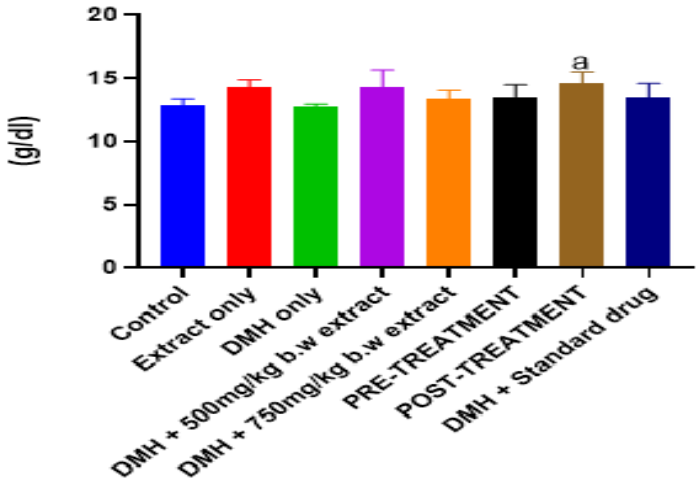

Figure 10. Haemoglobin level of rats in different groups. The letter 'a' signifies a statistically significant difference from the control group. Data are expressed as mean ± standard error of mean. MEFE increased the haemoglobin of treated animals; this was evident in group 2 animals and the animals that received post-treatment.

Discussions

Adenomatous polyposis coli (APC) is one of the components of the Wnt signaling pathway. Derangement in this pathway is a key event in the initiation of colorectal cancer. Mutation of APC, a tumor suppressor gene, predisposes individuals to colon cancer. It is believed that the main function of APC is the regulation of free β-catenin in concert with glycogen synthase kinase 3β (GSK-3β) (Perse and Cerar 2011). It has been found that half of the human colon tumors with intact APC protein have a mutation in the β-catenin gene (Tanaka et al. 2017).

Studies with the DMH rat model have detected mutations in the APC gene in colorectal epithelial lesions. In DMH/AOM-treated rats, up to 33% of colon tumors harbour APC mutations, but β-catenin mutations are more frequent than APC mutations (Takahashi et al. 2000, Bordonaro et al. 2016). When APC or β-catenin is mutated, β-catenin cannot be degraded but accumulates in the cytoplasm and translocates into the nucleus, where it binds to T-cell factor (TCF) and activates the Wnt target genes (Jin et al. 2008, Perse and Cerar 2011, Jin et al. 2022). In our study, in line with the previous findings (Yu et al. 2018, Salim et al. 2023), the relative expression of the APC gene in DMH-treated rats (grp3) was significantly decreased compared to the groups that were either cotreated, pretreated, or post-treated with *F.exasperata.* The high level of β-catenin that was expressed in group 3 might be a result of a decreased level of APC, which activates β-catenin phosphorylation, hence inhibiting its degradation (Shojaei-Zarghani et al. 2020). However, the level of expression of the β-catenin gene was lowered in the post-treated group. The results obtained in this study indicate that *F. exasperata* may be an effective chemopreventive agent for colon cancer through down-regulation of the GSk3β/β-catenin pathway.

The ability of *Ficus exasperata* leaf extract to suppress beta-catenin and uphold the APC gene may be a result of the phytochemicals present in this plant. Phytochemical screening reveals the presence of saponins, terpenoids, anthraquinones, flavonoids, alkaloids, carbohydrates, steroids, phenols, and tannins (Alli-Smith et al. 2018, Anigboro et al. 2019). These diverse phytochemicals contribute to the plant's anticarcinogenic properties. Anthraquinones have demonstrated anti-inflammatory, immunoregulatory, anti-hyperlipidemic, and anticancer effects in pharmacological studies (Lin et al. 2015, Cui et al. 2016, Abu et al. 2018, Yang et al. 2019, Wang et al. 2021). Flavonoids, abundant in the extract, exhibit antioxidant properties and

have demonstrated anticancer activities (Yahfoufi et al. 2018, Abotaleb et al. 2018, Chirumbolo et al. 2018, Rodriguez-Garcia et al. 2019). Saponins possess antimicrobial, antiviral, anti-inflammatory, anticancer, antioxidant, and immunomodulatory effects (Barbosa et al. 2014, Juang and Liang 2020). Terpenoids, known for their antitumor, anti-inflammatory, antibacterial, and antiviral properties, were also identified (Yang et al. 2020, Jahangeer et al. 2021). Plant alkaloids in the extract may contribute to its anti-inflammatory, antioxidant, and anti-mutagenic characteristics (Biribi 2018, Louis et al. 2019).

Lipid peroxidation of membranes is a process that plays an important role in initiating and mediating various health challenges, ranging from inflammation, cancer, and cardiovascular diseases, among others (Friedmann et al. 2019). In lipid peroxidation, carbon-carbon double bonds in lipids such as polyunsaturated fatty acids, phospholipids, glycolipids, and cholesterol undergo hydrogen abstraction and oxygen insertion in the allylic position, resulting in lipid peroxyl radicals and hydroperoxides (Stoyanovsky et al. 2019). The products of this lipid peroxidation are deleterious to DNA and other biological macromolecules (Olude and Omoregie 2024), which are considered oxidative stress biomarkers to specify the extent of cell injury (Valaei et al. 2022). High lipid peroxidation level in the colon tissue is directly related to the severity of DMH-induced lesions. (Jeong et al. 2025). Lipid peroxidation has been linked to one of the causes of colon cancer, which leads to the production of malondialdehyde. This product damages bio-cellular components, such as mutating DNA, signifying tumorigenicity (Yang et al. 2020). Administration of DMH elevated the level of lipid peroxidative product (MDA). MDA levels were significantly increased in group 3 compared to the other groups. A reduction in this lipid peroxidation marker was observed in groups treated with the plant extract. The post-treated and standard drug-treated groups had the lowest level of MDA. The first line of defense against oxidative stress is antioxidants. They break down free radicals into less reactive and harmless molecules (Balaji et al. 2015). Reduced glutathione level was reduced in group 3 compared to other groups, though not statistically significant compared to other treated groups. The decrease in the level might be due to its function in catalyzing and trapping free radicals.

Alterations and aberrations in haematological profiles by 1,2-dimethylhydrazine have been reported in previous studies. Most of the studies are unanimous on the fact that DMH suppresses haemoglobin level (Hb), as obtained in this study (Mishra et al. 2022, Salehi et al. 2022). Reduction in the level of heamoglobin (Hb) is a pointer to anaemia. RBCs are prone to the deleterious effects of radicals and carbonium ions that are produced during 1,2-dimethylhydrazine metabolism. These products formed from the activation of DMH from its proactive to its active state are released into biological systems, which elicits oxidative stress, DNA damage, and ultimately cell death. Thus, explaining the reduction of Hb in the DMH-treated group (Akinwunmi et al. 2023). The groups treated with the methanol extract of *Ficus exasperata* had a normal level of Hb and RBC, suggesting the plant extract may possess some anti-anaemic compounds. In the present report, WBC was also elevated after DMH exposure, as reported in a recent study (Mishra et al. 2022). The increase in the level of white blood cells recorded in this study might be as a result of inflammation, tumor production, and metastasis in DMH-induced rats, which provoked the immune system to produce a large amount of white blood cells (WBCs). However, the WBC differential pattern did not agree with previous studies. For instance, Mishra et al. (2022) reported a drastic decrease in neutrophils and lymphocytes, while Salehi et al. (2022) reported an increase in neutrophils. Neutrophil permeation has been described in human sporadic premalignant colonic adenomas (Mclean et al. 2011). Herein, it was demonstrated that DMH administration in the DMH-only group resulted in a significant increase in neutrophils, and eosinophils, and a slight increase in basophils. Simultaneous treatment with the extract enhances the immune system, with post-treatment being more beneficial. Methanol extract of *F. exasperata* may be beneficial in protecting blood cells against

anaemia and white blood cell pathologies during colon cancer proliferation. Molecular docking is recommended for further studies.

Conclusions

The study shows clearly that the plant extract is rich in numerous phytochemicals, which would have greatly contributed to one of the mechanisms through which this plant was able to suppress beta-catenin and uphold the expression of the tumor suppressor gene APC. In addition, this plant contains haematinic components and also boosts the immune system.

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