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Novel Metabolites from Crocodile Gut Microbiome

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14. ABSTRACT
The research work has yielded very exciting data that explains why gut microbiome of animals such as crocodile contribute to their hardiness, and this research has the potential to benefit human health and performance. The concept of using bacteria or their metabolites, and/or animal-derived molecules is not unusual, for example, “Mutaflor©” (specific strain of E. coli) is widely used as a probiotic and Captopril, a blood pressure drug was developed from snake venom. These are successful examples and the use of crocodile gut bacteria or their derived molecules to improve performance and wellbeing of humans is not far-fetched. Although the present research has identified the underlying molecular mechanisms in vitro, future in vivo studies are needed to elucidate their value in the rational development of innovative therapies against cellular stress/gut dysbiosis. Furthermore, the work has led to 9 quality peer-reviewed publications in leading journals during the current year of research activity.

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Key outcomes at the completion of second year of the AFSOR grant:

As a condition of receiving this generous support from the Air Force Office of Scientific Research, I am obliged to tender the following report as a result of this grant. The research work has yielded very exciting data that explains why gut microbiome of animals such as crocodile contribute to their hardiness, and this research has the potential to benefit human health and performance. The concept of using bacteria or their metabolites, and/or animal-derived molecules is not unusual, for example, "Mutaflor©" (specific strain of *E. coli*) is widely used as a probiotic and Captopril, a blood pressure drug was developed from snake venom. These are successful examples and the use of crocodile gut bacteria or their derived molecules to improve performance and wellbeing of humans is not far-fetched. Although the present research has identified the underlying molecular mechanisms *in vitro*, future *in vivo* studies are needed to elucidate their value in the rational development of innovative therapies against cellular stress/gut dysbiosis. Furthermore, the work has led to 9 quality peer-reviewed publications in leading journals during the current year of research activity.

Research Objectives

1. Investigate gut bacteria of crocodiles - completed
2. Identify the active conditioned medium of crocodile gut bacteria - completed
3. Identify the molecules using Liquid chromatography–mass spectrometry (LC-MS) - completed
4. Determine *in vitro* effects of active molecule(s) against a panel of bacteria - completed
5. Determine *in vitro* effects of active molecule(s) against a panel of human cells, to determine their effects on stress, ageing and longevity using biomarkers including oxidative stress, DNA methylation and senolytic drugs - completed

Summary of research:

1. Among a plethora of crocodile gut bacteria, two bacterial strains (CP27 and CP36) were isolated that showed promising bioactivities.
2. Using 16S rRNA sequencing, both bacterial strains, CP27 and CP36 were identified.
3. Metabolites from both bacterial isolates showed potent activities in a variety of bioassays (antibacterial, anti-inflammatory etc.).
4. When cerebrovascular endothelial cells were exposed to stress, the metabolites were more potent in inhibiting nitric oxide production than senolytic drugs (fisetin, quercetin).
5. Stress-induced release of pro-inflammatory cytokines (human interleukin 1 beta, human tumor necrosis factor alpha, and Prostaglandin E2) by cerebrovascular endothelial cells was inhibited by the metabolites.

6. Epigenetics studies using genome-wide DNA methylation analysis revealed that metabolites exhibit anti-inflammatory effects
7. Liquid chromatography-mass spectrometry (LC-MS/MS) of conditioned media of both CP27 and CP36 revealed the identity of metabolites including 3,4-dihydroxyphenylglycol, 5-methoxytryptophan, nifedipine, 4-chlorotestosterone-17-acetate, 3-phenoxypropionic acid, lactic acid, f-Honaucin A, l,l-Cyclo(leucylprolyl), 3-hydroxy-decanoic acid.
8. Nine peer-reviewed publications (annexure # 1).

Accomplishments:

The gut microbiome is well recognized to play a significant role in regulating the behaviour and health of its host. In recent years, there has been a marked increase in studies of the human microbiome. However, *Homo sapiens* are just one species among millions of other species and we are a relatively new addition to this planet. Other species such as crocodiles have shown the ability to adapt, evolve and survive successfully over millions of years, suggesting that we ought to learn from them. Having visited several crocodile sanctuaries, it was intriguing that crocodiles are routinely exposed to radiation, heavy metals, poor diet, pollution etc. and have survived the catastrophic Cretaceous-Tertiary extinction event, yet they can have a prolonged lifespan of up to 100 years. Even with exposure to stressful environments and carcinogenic materials, these species thrive under conditions that are considered detrimental to *Homo sapiens.* We hypothesized that crocodiles have developed mechanisms to achieve such longevity while surviving under stressful conditions. We speculate that their microbial gut flora may produce substances contributing to their "hardiness" and "longevity". Previously we characterized selected microbial gut bacteria colonizing the gastrointestinal tract of *Crocodylus porosus* (CP) using 16S rRNA sequencing. Metaproteomics mass spectrometric-based analysis confirmed the presence of most of the bacterial species identified using 16S rRNA sequencing analysis. Next, bacterial conditioned media containing gut microbial metabolites were prepared. These metabolites have been evaluated against various human cell lines for metabolic activities. Bioassay-guided testing of selected bacterial conditioned media using LC-TIMS-QTOF MS, revealed the identity of gut microbial metabolites. Among two bacterial conditioned media, i.e., CP27 and 36, the analyses resulted in 141 highly confidently (MS/MS) identified metabolites in both samples. The pairwise comparison of the two samples indicated that 109 metabolites change significantly between them (p <0.05). Among abundant metabolites more prevalent in CP27, there were 2-Methyl-4-nitroimidazole, N-Acetyl-L-tyrosine, Acetaminophen, Trans-Ferulic acid, N, N-Dimethylformamide, Pyrocatechol, Cyclohexanone, 3, 4-Dihydrozphenylglycol, Diphenhydramine, Melatonin, Gamma – terpinene. Whereas in CP36 samples the most abundant metabolites were Carbamazepin, Deoxyninosine, Cysteamine, Benzylnicotinate, 3-phenoxypropionic acid, Indole-3-carbinol, Benzaldehyde, Benzocaine, 2-Aminobenzoic acid, 3-Methylindole. Functional enrichment analysis of all identified metabolites with metabolite sets based on drug pathways showed that they were enriched for drug action of top ten pathways associating with enalapril metabolism pathway, diphenhydramine H1-Antihistamine action, enalarpil action pathway, benzocaine action pathway, mepivacaine action pathway, oxybuprocaine action pathways, nifedipine action pathway, propranolol action pathway, acetaminophen metabolism pathway, carbamazepine metabolism pathway. When cerebrovascular endothelial cells were exposed to stress, the metabolites were more potent in inhibiting nitric oxide production than senolytic drugs (fisetin, quercetin). Stress-induced release of pro-inflammatory cytokines (human interleukin 1 beta, human tumor necrosis factor alpha, and Prostaglandin E2) by cerebrovascular endothelial cells was inhibited by the metabolites. Overall, crocodile gut bacteria, CP27 and CP36 contain metabolites to exact their effects [(3,4-

dihydroxyphenylglycol, 5-methoxytryptophan, nifedipine, 4-chlorotestosterone-17-acetate, 3-phenoxypropionic acid, lactic acid, f-Honaucin A, l,l-Cyclo(leucylprolyl), 3-hydroxy-decanoic acid etc.]. Epigenetics studies using genome-wide DNA methylation analysis revealed that metabolites exhibit anti-inflammatory effects, indicative of their potential in providing protection against cellular stress. Future *in vivo* studies are needed to elucidate their value in the rational development of innovative therapies against cellular stress/gut dysbiosis.

Major activities:

Methods:

Cerebrovascular endothelial cells cultivation

Human cells were grown prior to the experiments. Human cerebral microvascular endothelial cells (HBEC-5i) (T0005011, Addexbio) were grown in Dulbecco's Modified Eagle Medium; Nutrient Mixture F12 (DMEM/F12) with L-glutamine, glucose and sodium pyruvate 500 mL (Addexbio). The cells were incubated at 37 °C in cell culture incubator with 5% CO_2 and humidity. Upon confluency (approximately 1 x 10^6), the cells were trypsinized using Trypsin-EDTA and cell suspension were centrifuged at 2,500 × g for 5 minutes. Cell pellets was reconstituted in their respective media and seeded in 96-well plates and 24-well plates. The established cell monolayer was then employed in various experiments.

Nitric oxide assays

Nitric oxide (NO) assays were performed to measure the conversion of nitric oxide to nitrite ions using modified Griess reagents (Bryan and Grisham 2007; Sarkar et al. 2015). Briefly to measure NO in human cells, HBEC-5i were grown to confluency at 37 °C with 95% humidity and 5% CO_2. The established cell monolayer was treated with senolytic drugs (fisetin, quercetin and etoposide at 100 µg/mL), as well as bacterial metabolites (100 µL). For controls, cells incubated with taxol (50 µg/mL) and cells incubated with N^G-nitro-L-arginine methyl ester, hydrochloride in serum-free RPMI were taken as positive and negative controls respectively. After this incubation, cell supernatants were collected after centrifugation and 100 µL of these supernatants were mixed with 100 µL of modified Griess reagents (1%). Finally, the plate was incubated for 30 min in the dark and the color development corresponding to NO level was assessed at 540 nm with a microplate reader (Bryan and Grisham 2007; Sarkar et al. 2015).

In some experiments, cells were stimulated with 50 µg/mL of taxol at different time points (*i.e.,* 3 h, 6 h and 12 h). After each incubation time, 100 µL of cell supernatants were combined with 1% modified Griess reagents *i.e.,* 100 µL. The plates were incubated for half an hour in the dark to develop color. Microplate reader was used to evaluate the NO level at 540 nm (Bryan and Grisham 2007; Sarkar et al. 2015).

To determine whether NO release in response to stress can be inhibited, HBEC-5i cells were pre-treated with metabolites and senolytic drugs (100 µg/mL), in combination and alone, in a 24-well plate for 2 h. Next, the cells were stimulated with 50 µg/mL of taxol and NO release was measured as described above.

Proinflammatory cytokines including human interleukin 1 beta (IL-1β), human Tumor necrosis factor alpha (TNF-α) and Prostaglandin E2 (PGE2

Enzyme linked immunosorbent assays (ELISA) were performed to quantify proinflammatory markers including human interleukin 1 beta (IL-1β) (Kolb et al. 2001), human Tumor necrosis factor alpha (TNF-α) (Grebenchtchikov et al. 2005) and Prostaglandin E2 (PGE2) (Ke et al. 2016) in human cerebrovascular endothelial cell culture supernatants treated with metabolites derived from CP27, and CP36. Briefly, HBEC-5i were seeded in a 24-well plate and when the confluency reached up to 80-90%, the cell monolayer was treated with metabolites of CP27, CP36 and *E. coli* K-12. After this incubation, cell supernatants were

collected and further centrifuged for 10 minutes at 2000× g. On the other hand, all the buffers, standards and antibodies cocktail were prepared according to the manufacturer's guidelines. Standards were two-fold serially diluted in phosphate buffered saline to prepare standard curve for each assay. For assays, 50 µL of samples/standards to corresponding well previously coated with specific antibodies were added. Next, for TNF-α and Human IL-1β, detector and capture antibodies were mixed together and 50 µL of the mixtures were added. The plates were covered with a sealer and were incubated at room temperature using a plate shaker at 400 x rpm for 60 minutes and 2 h respectively. For PGE2, biotin-labeled antibody (50 µL) was added to the corresponding well and the plate was incubated for 45 minutes on a shaker set at 400 x rpm and at room temperature. After each corresponding incubation, the reaction mixtures (samples/standards and antibody mixture) were discarded from each well and washed three times with 1X wash buffer. After the last wash, plates were inverted and blotted against a clean paper towel to ensure complete removal of wash buffer. After washing, 100 µL of 3,3´,5,5´-tetramethylbenzidine (TMB) solution was added to each well of IL-1β and TNF-α plates and the plates were incubated for 10 minutes on a shaker with 400 rpm. Finally, 100 µL of stop solution was added, plates were shaken for 1 minute and readings were taken at 450 nm using microplate reader. For PGE2, after washing 100 µL HRP-streptavidin conjugate was added to each well and the plate was further incubated for 30 minutes at 37°C. Next, solutions were discarded and the plate was washed 5 times with washing buffer. TMB solution (90 µL) was added to each well, and the plate was incubated in the dark for 15 minutes. Finally, 50 µL of stop solution was added and optical density was recorded at 450 nm by microplate reader.

Whole genome DNA methylation analysis

Global epigenetic patterns and variations among different samples were characterized using whole genome bisulfite sequencing (WGBS) to study DNA methylation as stated previously (Lee et al., 2017; Meyer et al., 2022). Briefly, Illumina WGBS analysis was used to characterize genome-wide patterns of DNA methylation in treated and untreated cells, in an effort to further our understanding of epigenetic regulation in cells under stress and normal conditions. Briefly, genomic DNA was collected using a Qiagen AllPrep Mini Kit. Isolated DNA was be eluted in TE buffer, validated for quality and quantity using UV spectrophotometry, and stored at -80°C. DNAs with an OD260/280 ratio between 1.75 and 1.85 was used for further investigations. 100 ng of genomic DNA was fragmented to 350 bp with the Covaris S220 Focused-ultrasonicator (Covaris). DNA was bisulphite treated using the EZ DNA Methylation-Gold™ Kit (Zymo Research). WGBS libraries were prepared using the Accel-NGS® Methyl-Seq DNA Library Kit (Swift Biosciences) following the recommended instructions and five PCR cycles for the final library amplification. The WGBS libraries were sequenced using the NovaSeq platform (Illumina) and an S4 flowcell with 1% PhiX spike-in (Illumina). All samples were spiked with 0.25% unmethylated lambda phage DNA (Promega) before fragmentation. The unmethylated lambda phage DNA serves as bisulphite conversion control. Base-calling was performed by analysing the raw sequencing output, bcl-files, with bcl2fastq2 ver. 2.20 (Illumina). Adapter trimming was performed with AdapterRemoval ver. 2.3.2 (parameters: – qualitybase 33, – minquality 30, – minlength 30, – trimqualities, and – trimns). Reads were aligned to the genome, and duplicates were removed using Bismark version 0.22.4. Methylation was called using the bismark_methylation_extractor with default parameters. All CpG sites with ≥ 1 read were called. CpG sites were annotated to a CpG island, a CpG island shore, a CpG island shelve, or open sea (other locations in the genome) based on the GENCODEvM25 gene annotation file from SeSAMe.

Statistical analysis

For statistical analysis and data visualization, the Graph Pad Prism version 8.0.2 (GraphPad Software; San Diego, CA, USA) was employed. To compare the different treatments

with their corresponding controls, a two-sample t-test with a two-tailed distribution was conducted. The results are presented as the mean ± standard error, calculated from multiple replicated experiments.

Ultra-performance Liquid Chromatography Tandem Mass Spectrometry (HPLC-MS/MS

The MS analysis was performed using a TimsTOF (Bruker, Darmstadt, Germany) with Apollo II electrospray ionization (ESI) source as described in our recent studies (Siddiqui et al., 2023). The drying gas was set to flow at 10 L/min and the drying temperature to 220°C and the nebulizer pressure to 2.2 bar. The capillary voltage was 4500 V and the end plate offset 500V. For proteomics, the scan range was 150 – 2200 m/z, and for metabolomics 20-1300 m/z. The instrument was operated in auto-MS/MS mode and for proteomics the collision energy was set to 7 eV using a fixed cycle time of 3s and a minimum relative intensity threshold of 500 counts per thousand and a target intensity of 10,000 cps and analyte charge was required to be $2 \geq x \geq 5$. For metabolomics the collision energy was set to 20 eV, the cycle time to 0.5 seconds with a relative minimum intensity threshold of 400 counts per thousand and target intensity of 20,000. MetaboScape® 4.0 was used for metabolite processing (Bruker Daltonics). To detect which metabolites showed significantly altered abundance for each treatment, a two-tailed independent students t-test was utilized for comparing the effect of each drug to that of DMSO. Only identified features with $p<0.05$ and a $\log_2$(fold-change) greater than 1 were considered further, excepting that the fold-change requirement was not necessary for inclusion in pathway enrichment analysis (see below). In addition, a one-way analysis of variance (ANOVA) was utilized to compare the effects of the treatments and investigate their interaction across the three groups. The threshold for significance was again set with $p<0.05$. Gene Ontology (GO) term and pathway enrichment analyses were performed using metaboanalyst (https://www.metaboanalyst.ca). All data, including the raw QGD files, have been deposited in the Metabolomics Workbench repository (https://www.metabolomicsworkbench.org/). RAW data files were analysed with MaxQuant version 1.6.17.0 [19] for protein and peptide identification using the Andromeda search engine and against several bacterial species. For the MS/MS database search, the default parameter settings were applied, with carbamidomethylation of cysteine residues set as fixed modification and acetylation of protein N-termini and methionine oxidation assigned as variable modifications. Peptide spectral matches (PSMs) were filtered with a 1% false discovery rate (FDR) and a 20-ppm precursor mass tolerance. Protease The default trypsin/P enzymatic cleavage rule was used for *in silico* digestion and the MaxLFQ algorithm was used for label-free quantitation (LFQ).

Significant results and key outcomes:

Microbiome composition likely contributes to the health and well-being of C. porosus

In essence, the research proposed that gut bacteria of crocodiles play a role in the hardiness and well-being of its host. Our earlier work using metagenomics analysis, revealed high bacterial diversity in the order of Proteobacteria, Bacteroidota, Firmicutes, Actinobacteriota, Desulfobacterota, Synergistota, Chloroflexi, and others (Fig. 1), which may potentially prevent intestinal dysbiosis and its systemic consequences suggesting that *C. porosus* microbiome has the potential of yielding novel bioactives to endure harsh conditions, as well as demonstrating increased longevity and reduced cellular senescence.

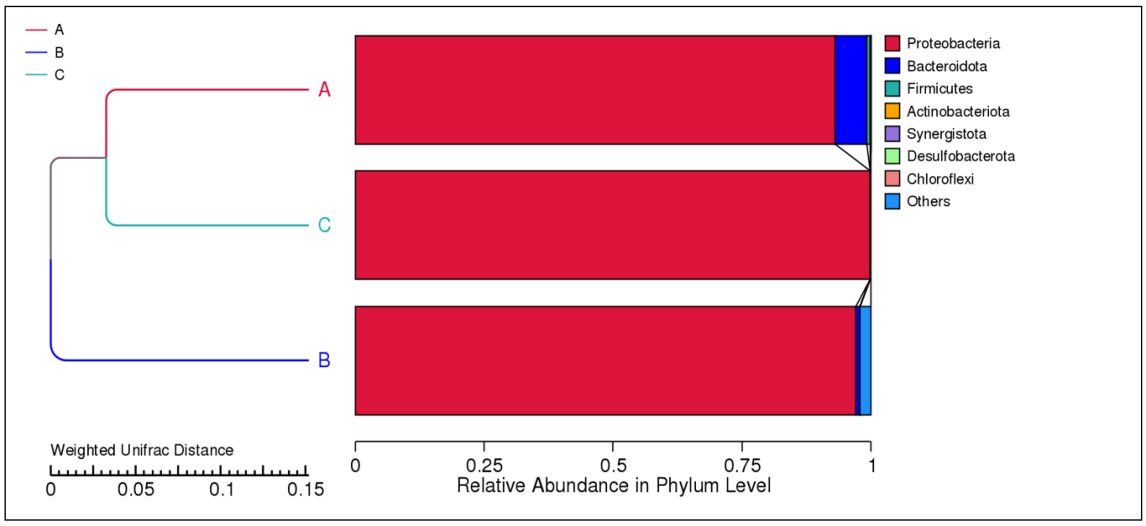

Figure 1. Unweighted Pair-group Method with Arithmetic Mean (UPGMA) cluster tree based on Weighted Unifrac distance between microbial communities of the oral cavity (A), small intestine (B) and large intestine (C) of the *C. porosus.*

Metabolites from CP27 and CP36 inhibited nitric oxide production in cerebral microvascular endothelial cells, in response to stress

Nitric oxide assays were conducted using modified Griess reagent to quantify the NO level in human cells. The results revealed that upon incubation, the metabolites of CP27 and CP36 and senolytic drugs (fisetin and quercetin) inhibited the NO production in human cells (Fig. 2). Among the senolytic drugs, fisetin and quercetin inhibited 70% (P = 0.004) and 64% (P = 0.029) of NO production when compared with the control (*i.e.*, taxol 100%; actual mean value =1.383 ± 0.04). Similarly, treatment with metabolites of CP27 and CP36 generated only 28% (mean value = 0.392 ± 0.05, P = 0.031) and 24% (mean value = 0.337 ± 0.02, P = 0.005) NO respectively in endothelial cells.

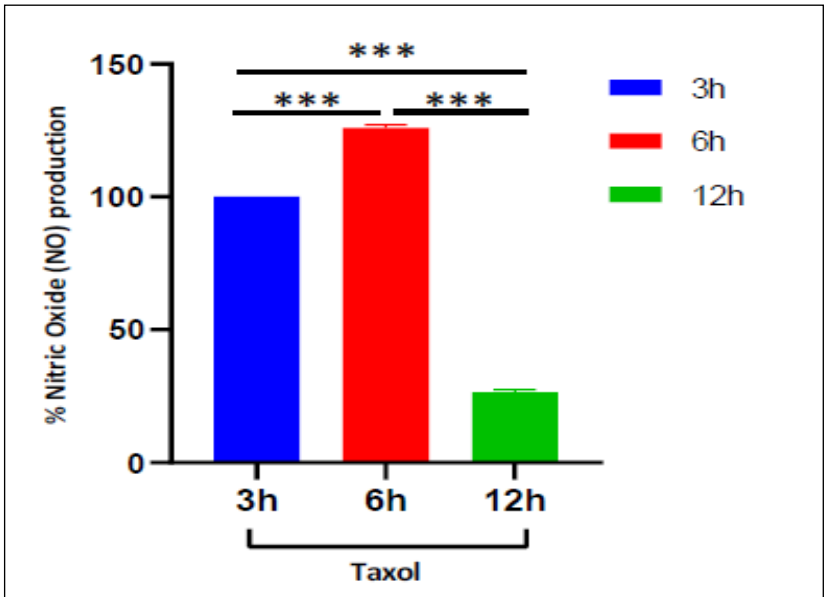

Figure 2. Human brain microvascular endothelial cells were treated with taxol at different time points and nitric oxide release was measured. Note that the highest level of NO was observed at 6 h of incubation (actual mean 1.513 ± 0.006). Similarly, the actual mean value for NO release at 3 h and 12 h were 1.202 ± 0.006 and 0.319 ± 0.008). The data are presented as the mean ± standard error of experiments performed 3 times in duplicates. (*) indicates $p \leq 0.05$.

Next, human cerebral microvascular endothelial cells were treated with taxol for 3, 6 and 12 h. The results indicated that incubation of cells with taxol resulted in NO release in cerebral microvascular endothelial cells. However, the highest percentage of NO production was recorded after 6 h of incubation (Fig. 2).

To determine whether taxol-mediated NO release can be blocked, human cerebrovascular endothelial cells were pre-treated with senolytic drugs and metabolites. The results revealed that the pre-treated endothelial cells with metabolites of CP27 and CP36 remarkably reduced NO production after 3 h ($P \leq 0.01$) (Fig. 3a). When compared to positive control *i.e.*, taxol alone (considered as 100%; mean value = 1.202 ± 0.008), metabolites of CP27 and CP36 reduced NO to 58% (mean value = 0.697 ± 0.01, P = 0.005) and 54% (mean value = 0.645 ± 0.009, P = 0.011) respectively. The metabolites of CP27 and CP36 alone generated 33% (mean value = 0.404 ± 0.02, P = 0.013) and 18% (mean value = 0.227 ± 0.006, P = 0.003) NO (Fig. 3a) while metabolites of *E. coli* K-12 failed to abridge the NO production after taxol treatment (Fig. 3a). Similarly, fisetin and quercetin lowered NO level to 77% (mean value = 0.940 ± 0.01, P = 0.049) and 74% (mean value = 0.907± 0.008, P = 0.032) (Fig. 3b). Fisetin and quercetin alone produced 47% (mean value = 0.570 ± 0.012, P = 0.014) and 40% (mean value = 0.489 ± 0.002, P = 0.006) NO gas.

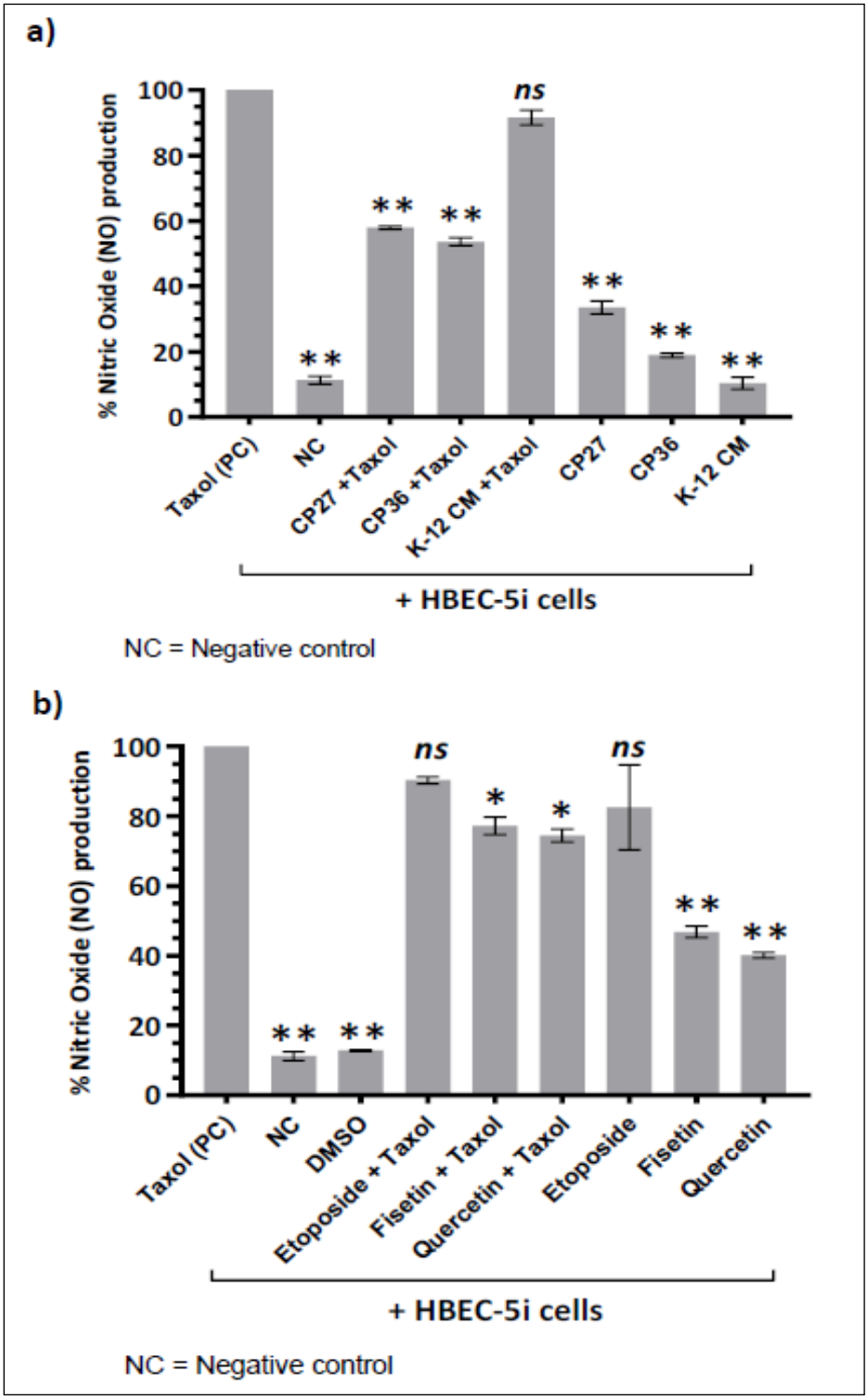

Figure 3. Senolytic drugs significantly reduced taxol-mediated NO production. Note that metabolites of CP27 and CP36, as well as senolytic drugs (fisetin and quercetin) reduced taxol-mediated NO release in cerebrovascular endothelial cells. The data are presented as the mean ± standard error. (*) and (**) indicates $p \leq 0.05$ and $p \leq 0.01$ respectively.

At 6 h of taxol treatment, metabolites of CP27 and CP36 significantly inhibited the NO liberation while metabolites of *E. coli* K-12 did not show inhibitory effects ($P \leq 0.01$) (Fig. 4a). Metabolites from CP27 inhibited 54% (mean value = 0.702 ± 0.007, P = 0.002) while CP36 inhibited 70% (mean value = 0. 469 ± 0.04, P = 0.017) NO production (Fig. 4a). Interestingly after 6 h of taxol treatment, the highest amount of NO was produced but both the metabolites (*i.e.,* CP27 and CP36) inhibited more NO as compared to 3 h of incubation, as depicted in Fig 3. Metabolites of CP27 and CP36 alone further reduced NO production up to 23% (mean value = 0.356 ± 0.01, P = 0.005) and 15% (mean value = 0.239 ± 0.01, P = 0.005) respectively (Fig. 4a). Fisetin and quercetin reduced NO production up to 65% (mean value = 0.973 ± 0.01, P = 0.002) and 64% (mean value = 0.964 ± 0.02, P = 0.029) after being treated with taxol for 6 h, while the drugs alone produced 36% (mean value = 0.535 ± 0.03, P = 0.016) and 33% (mean value = 0.502 ± 0.01, P = 0.004) NO in human cerebrovascular endothelial cells (Fig. 4b). These are remarkable findings and clearly showed that metabolites derived from crocodile gut bacteria inhibit nitric oxide production by human cells.

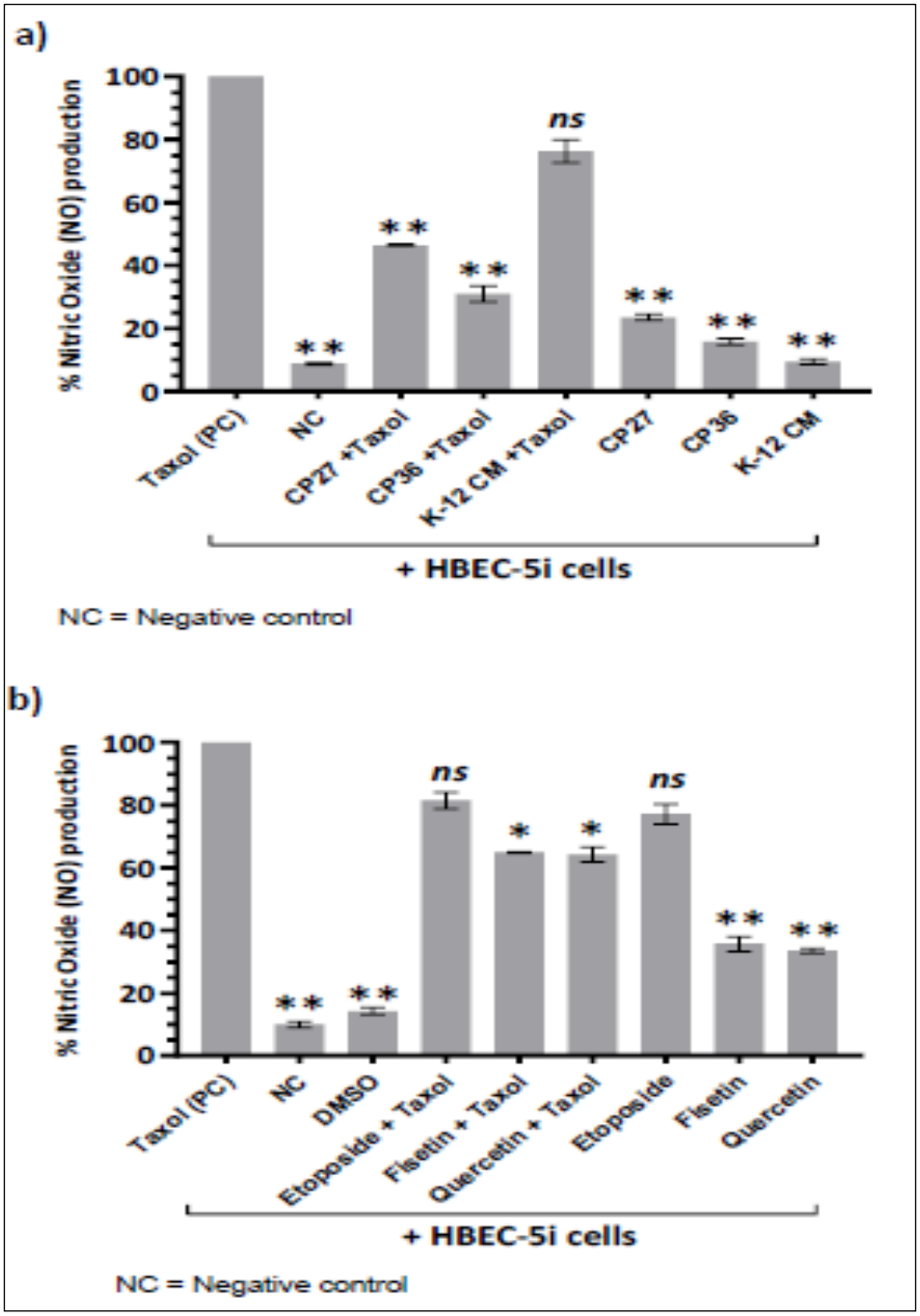

Figure 4. Note that metabolites of CP27 and CP36, and senolytic drugs (fisetin and quercetin) reduced taxol-mediated NO release in endothelial cells. The data are presented as the mean ± standard error of several independent experiments performed in duplicate. (*) and (**) indicates $p \leq 0.05$ and $p \leq 0.01$ respectively.

Endothelial cells treated with metabolites exhibited reduced level of pro-inflammatory cytokines release in response to stress

ELISA assays revealed that taxol treatment produced highest level of IL-1β proinflammatory cytokine (Fig. 5a and 5b). Pre-treatment with metabolites of CP27 and CP36 reduced taxol-mediated IL-1β release. Cells pre-treated with metabolites of CP27 and CP36, followed by stimulation with taxol, reduced IL-1β release to 469 pg/mL and 441 pg/mL IL-1β respectively (Fig. 5b). The metabolites of CP27 and CP36 alone generated 298 pg/mL and 395 pg/mL human IL-1β (Fig. 5b). In contrast, metabolites of *E. coli* K-12 alone produced only 140 pg/mL while after stimulation with taxol the cytokines drastically increased to 923 pg/mL (Fig. 5b).

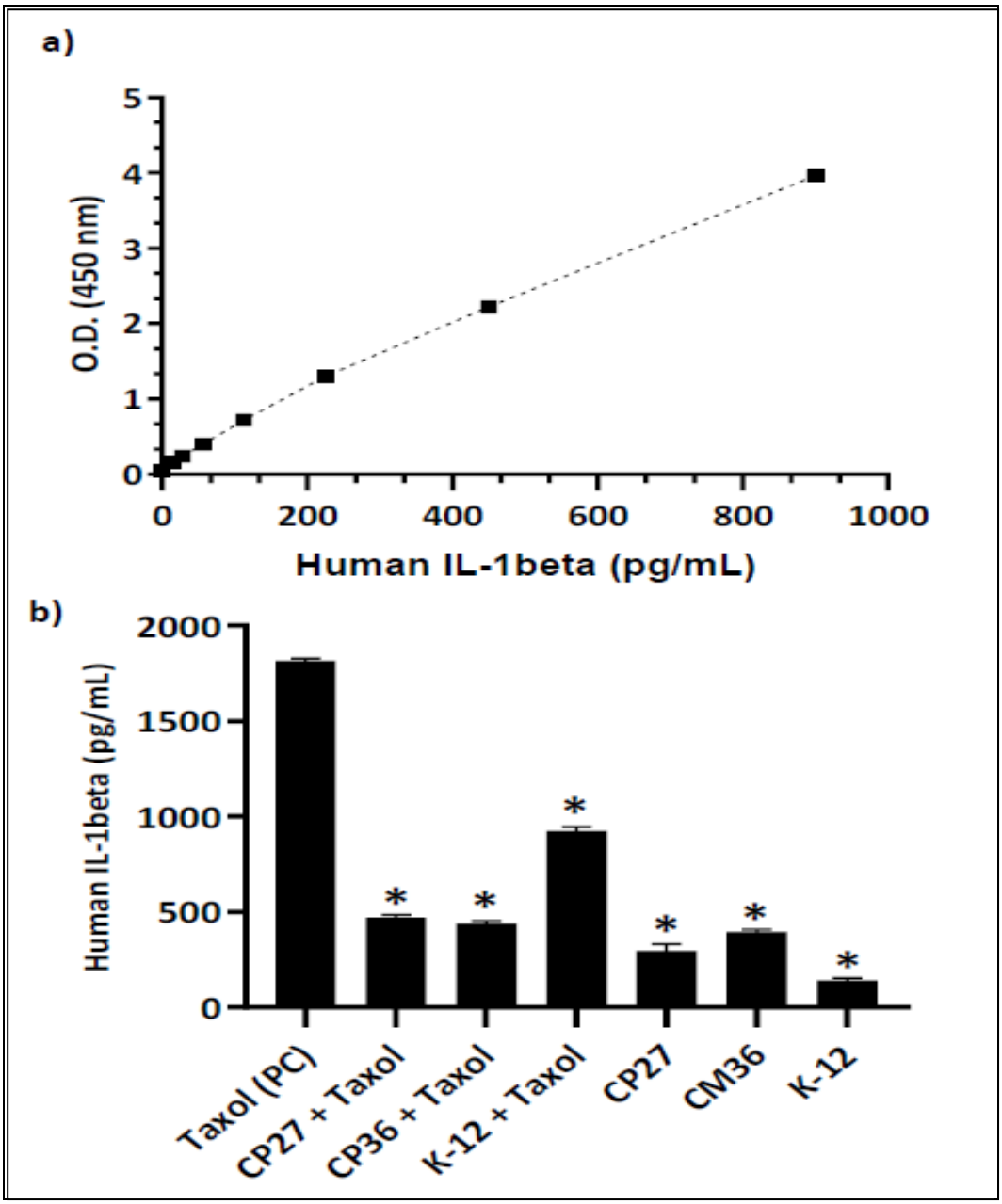

Figure 5. Estimated concentrations of human IL-1 beta in human endothelial cells treated with metabolites of CP27, CP36, K-12 conditioned media and taxol stimulated cells. The IL-1beta concentrations were determined, estimated from the IL-1beta standard curves, and sample dilution was adjusted. a**).** IL-1 beta standard curve prepared in sample diluent. **b).** Quantification of human IL-1β in treated human cells supernatants. The data are presented as the mean ± standard error. (*) indicates $p \leq 0.05$.

Similar findings were observed with TNF-α and PGE2 (Fig. 6 and 7). While metabolites of CP27 and CP36 alone produced minimal TNF-α and PGE2 pro-inflammatory cytokines. Intriguingly after stimulation with taxol, the cytokine release was reduced by pre-treatment with metabolites of CP27 and CP36 when compared to the positive control i.e., taxol alone (100%) (Fig. 6 and Fig. 7). Metabolites of CP27, CP36 and *E. coli* K-12 conditioned media, generated 297 pg/mL,241 pg/mL and 287 pg/mL TNF-α respectively (Fig. 6) whereas CP27 and CP36 significantly reduced TNF-α production when treated with taxol (Fig. 6).

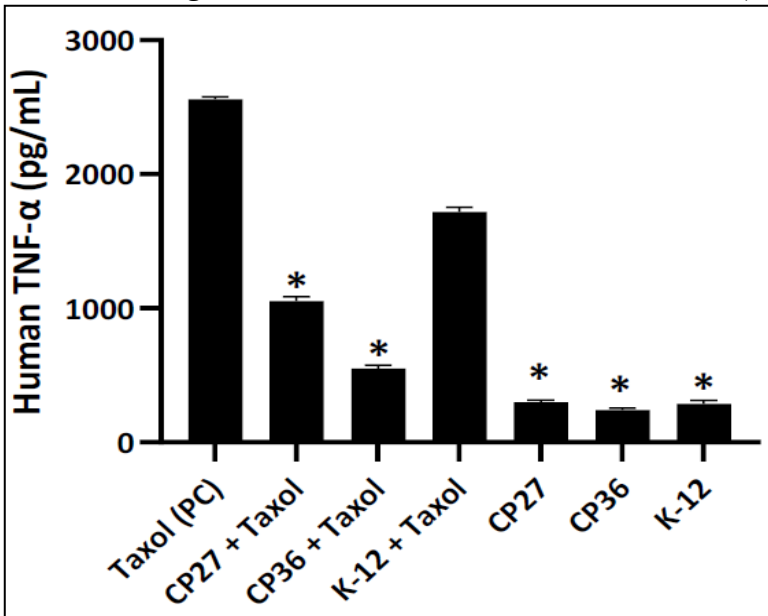

Figure 6. Estimated concentrations of human TNF-α in endothelial cells treated with metabolites and taxol stimulated cells. The data are presented as the mean ± standard error. (*) indicates $p \leq 0.05$.

For PGE2, similar results were observed. Pre-treatment of human cerebrovascular endothelial cells using metabolites of CP27 and CP36 inhibited pro-inflammatory cytokine PGE2 as shown in Fig. 7. In contrast, metabolites of K-12 showed no effect. Metabolites of CP27 and CP36 pre-treated cells significantly regulated PGE2 cytokines when stimulated with taxol (Fig. 7).

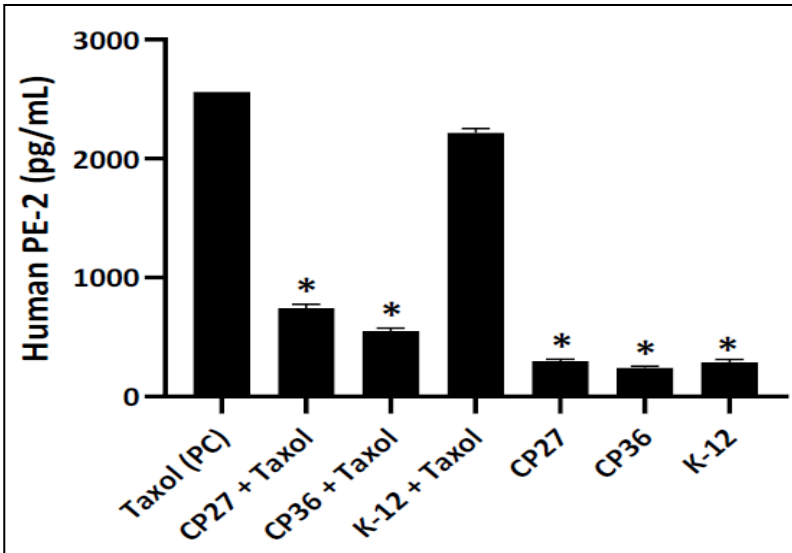

Figure 7. Estimation of human PGE2 in treated human cells supernatants. The data are presented as the mean ± standard error. (*) indicates $p \leq 0.05$.

Whole genome DNA methylation analysis revealed differentially expressed genes

An overall presentation of differential gene expression analysis data in response to metabolites of CP27 and CP36 exposure was determined. After metabolite of CP27 administration, blue dots (239923) represent the number of significant DEGs between treated and untreated group and the green dots represent down-regulated genes and red dots represent up-regulated genes respectively. A total of 1066 genes were up-regulated and 621 genes were down regulated when cells were treated with metabolites of CP27. When treated with metabolites of CP36, the number of total DEGs were 3934, out of which 2238 were up-regulated while 1696 were down-regulated.

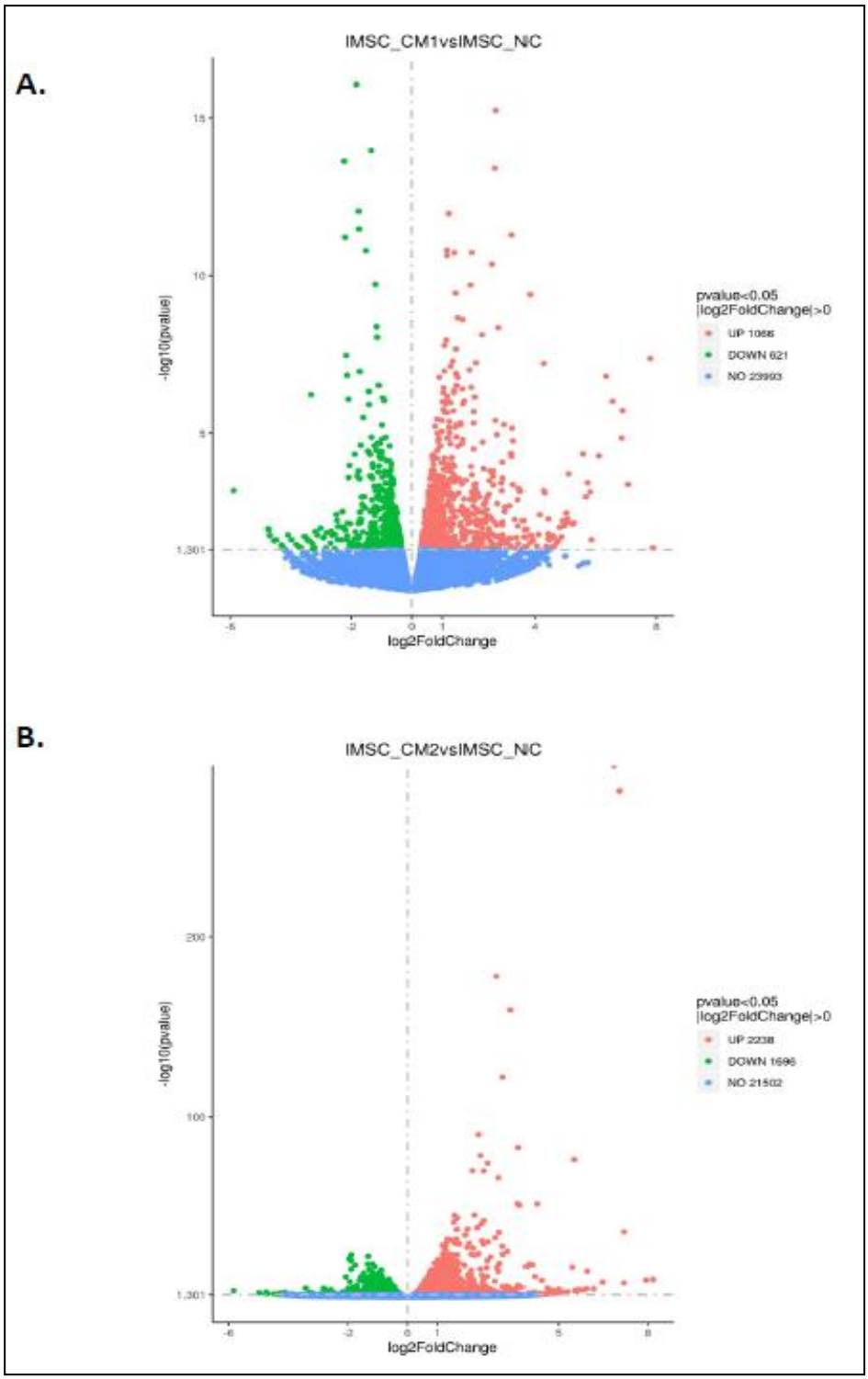

Figure 8. Volcano plots demonstrating overall distribution of Differentially Expressed Genes (DEGs). The log2 FoldChange indicates the mean expression level for each gene. Each dot represents one gene. A. After metabolite of CP27, blue dots (239923) represent no significant DEGs between NC group and green dots represent down-regulated genes and red dots represent up-regulated genes respectively; A total of 1066 genes were up-regulated and 621 genes were down regulated. B. when treated with metabolites of CP36, the number of total DEGs were 3934, out of which 2238 were up-regulated while 1696 were down-regulated.

Using whole genome DNA methylation analysis revealed Top 20 enriched GO terms and KEGG pathways

Top 20 enriched GO terms and KEGG pathways in cells treated with metabolites of CP27 and CP36 compared to cells alone. The Go term analysis revealed that majority of the genes are involved in the regulation of the cellular pathways such as cell to cell adhesion, cell locomotion, migration, motility and angiogenesis etc. (Fig. 9A). The KEGG pathway further confirmed that these DEGs were involved mainly in P13Akt signalling pathway which a key role in cell survival, proliferation and metabolism (Fig. 9B). These genes are also involved in human herpesvirus infection i.e., kaposi sarcoma associated herpesvirus infection. Similarly,

metabolites of CP36 treatment revealed that the DEGs play a role in blood vessel morphogenesis, cell migration, wound healing, angiogenesis, GTPase activity and cellular response to interferon type 1 (Fig. 9C). The KEGG analysis showed that these genes are further involved in the viral infection such as human cytomegalovirus, influenza A, Epstein Bar virus etc., cytokine-cytokine receptor interaction, calcium signalling pathway and TNF signalling pathways (Fig. 9D).

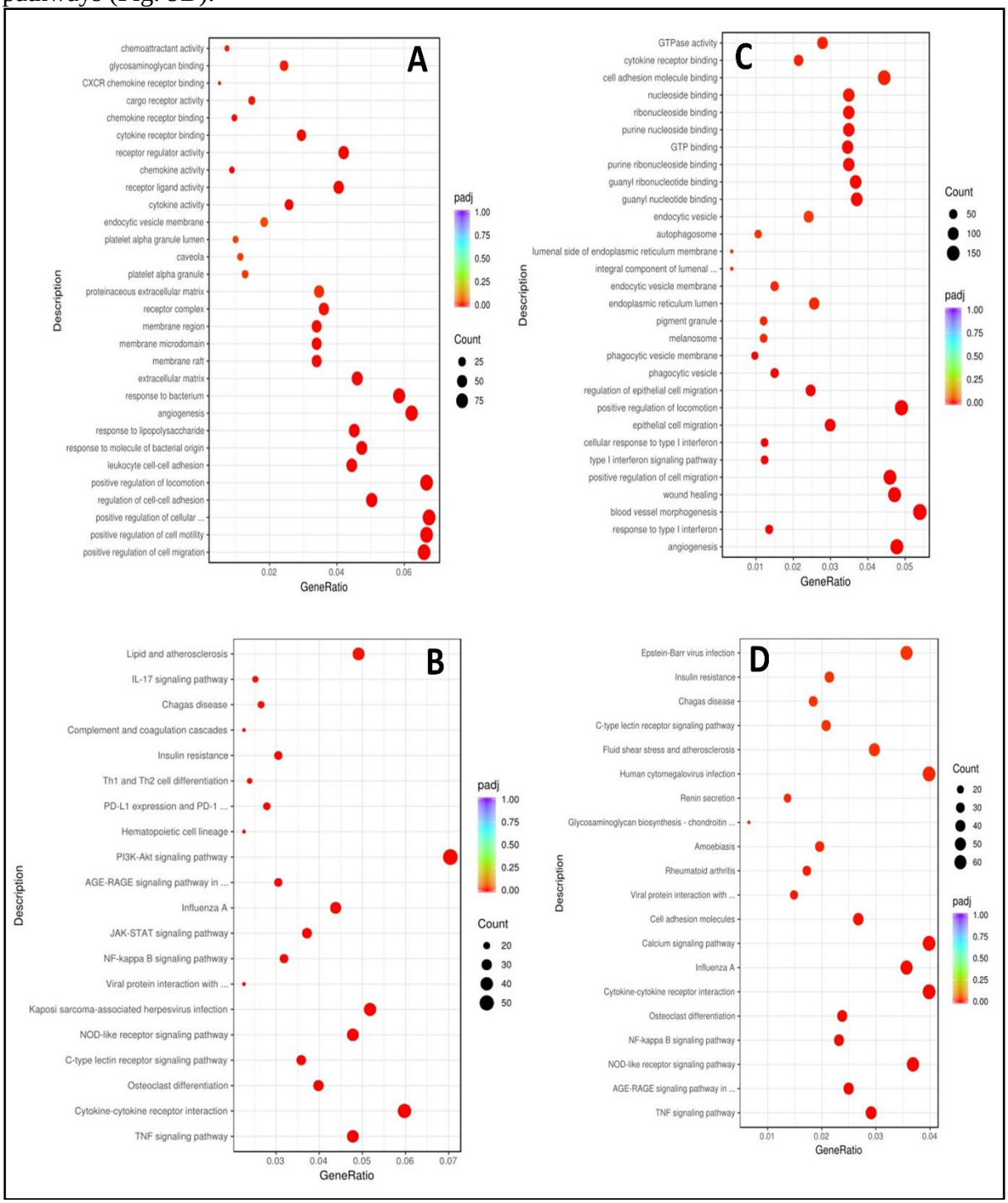

Figure 9. Top 20 enriched GO terms and KEGG pathways. The Go term analysis revealed that majority of the genes are involved in the regulation of the cellular pathways such as cell to cell adhesion, cell locomotion, migration, motility and angiogenesis, role in blood vessel morphogenesis, cell migration, wound healing, angiogenesis, GTPase activity and cellular response to interferon type 1. etc. The KEGG pathway further confirmed that these

DEGs were involved mainly in P13Akt signalling pathway which a key role in cell survival, stress, proliferation and metabolism, as well as in the viral infection such as human cytomegalovirus, influenza A, Epstein Bar virus etc., cytokine-cytokine receptor interaction, calcium signalling pathway and TNF signalling pathways.

Mass spectrometry revealed several secondary metabolites

We used an untargeted LC-MS/MS-based metabolomics analysis and searched against the pharmacology library. All the experiments were carried out in at least three biological replicates, and each metabolite extract was analysed in duplicate by LC-TIMS-QTOF MS. The analyses resulted in 141 highly confidently (MS/MS) identified metabolites in both sample CP27 and CP36. The pairwise comparison of the two samples indicated that 109 metabolites change significantly between them (p <0.05), with 47 and 62 were more abundant in CP27 and CP36, respectively. Among abundant metabolites more prevalent in CP27 there were 2-Methyl-4-nitroimidazole, N-Acetyl-L-tyrosine, Acetaminophen, Trans-Ferulic acid, N,N-Dimethylformamide, Pyrocatechol, Cyclohexanone, 3,4-Dihydrozphenylglycol, Diphenhydramine, Melatonin, Gamma –terpinene (see Figure 10). Whereas metabolites of CP36 samples the most abundant metabolites were Carbamazepin, deoxyninosine, Cysteamine, Benzylnicotinate, 3-phenoxypropionic acid, Indole-3-carbinol, Benzaldehyde, Benzocaine, 2-Aminobenzoic acid, 3-Methylindole (Fig. 10).

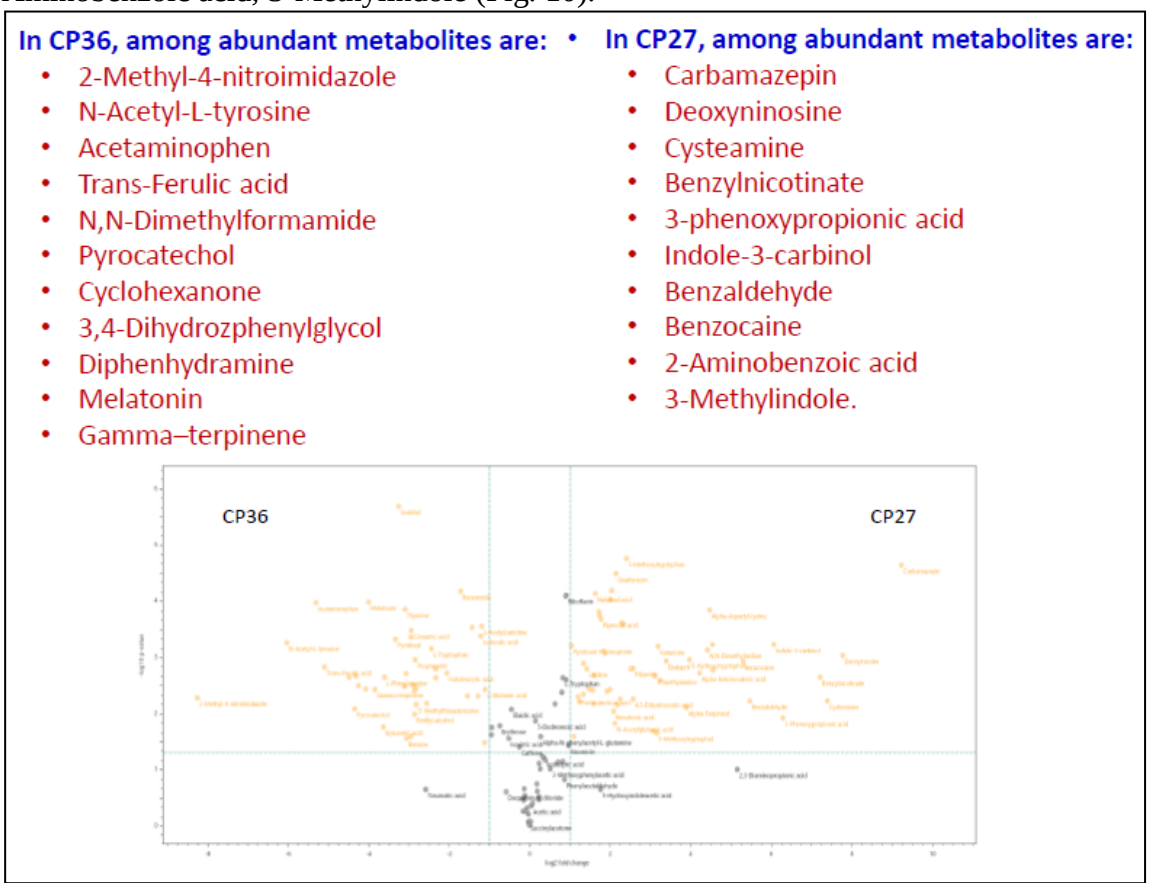

Figure 10. Liquid chromatography-mass spectrometry (LC-MS/MS) revealed the identity of metabolites including 3,4-dihydroxyphenylglycol, 5-methoxytryptophan, nifedipine, 4-chlorotestosterone-17-acetate, 3-phenoxypropionic acid, lactic acid, f-Honaucin A, l,l-Cyclo(leucylprolyl), 3-hydroxy-decanoic acid.

Functional enrichment analysis of all identified metabolites with metabolite sets based on drug pathways revealed association with Enalapril metabolism pathway; Diphenhydramine H1-Antihistamine action; Enalarpil action pathway; Benzocaine action pathway; Mepivacaine

action pathway; Oxybuprocaine action pathways; Nifedipine action pathway, propranolol action pathway; Acetaminophen metabolism pathway; Carbamazepine metabolism pathway (Fig. 11).

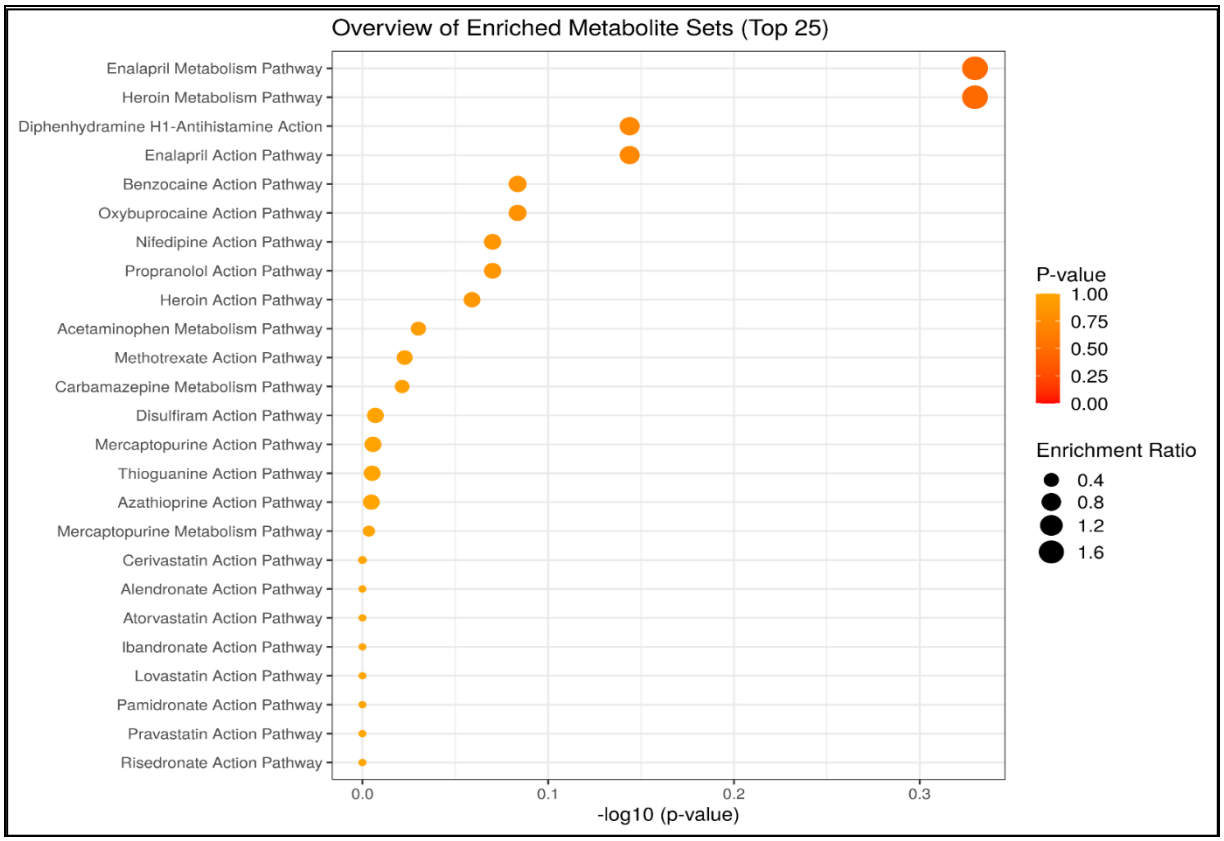

Fig. 11. Functional enrichment analysis of all identified metabolites with metabolite sets based on drug pathways showed that they were enriched for drug action of top ten pathways associating with enalapril metabolism pathway, diphenhydramine H1-Antihistamine action, enalarpil action pathway, benzocaine action pathway, mepivacaine action pathway, oxybuprocaine action pathways, nifedipine action pathway, propranolol action pathway, acetaminophen metabolism pathway, carbamazepine metabolism pathway.

Discussion

The use of long-lived and hardy animals, such as crocodiles, is an important area worthy of investigation as it may present unique opportunities to comprehend and potentially utilize their defense systems against cellular stressors, improve longevity and reduce ageing (Siddiqui et al. 2021). Given their status as “living fossils,” crocodiles are unquestionably a fascinating species in this sense (Stockdale and Benton 2021; Siddiqui et. al. 2021). It is now known that crocodiles, have a distinctive gut microbiome, which may almost certainly affect their capacity to manufacture antimicrobial peptides as well provide their resilience to disease and their longevity (Khan et al. 2021; Willson et al. 2019). Since it is apparent that in humans, 70–80% of immune cells are located in the gut, illustrating a deep interaction with the gut microbiota; it is likely that the robust immune system of crocodiles is a critical factor impacting their capacity to adapt to their environment stressors (Laissue et al. 1993; Siddiqui et al. 2022). In this regard, we previously isolated bacterial isolates from the crocodile gut flora (Khan et al. 2021), and herein evaluated their ability to protect against cellular stressor mediated by NO production and proinflammatory cytokines.

Stress physiology and stress-related disease processes involve NO, and NO is an inflammatory mediator produced in response to stress (Esch et al. 2002; Pagliaro 2003). Thus, NO and stress are closely connected but the process is multifactorial (Esch et al. 2002). In the present work, the ability of the metabolites from the crocodile gut bacteria to mediate NO production in response to cellular stress, as well as the levels of pro-inflammatory cytokines (IL-1β, TNF-α, PGE2) was determined. The senloytic compounds fisetin and quercetin were also utilized for comparison, as senolytic drugs are known to selectively clear senescent cells, and are thought to be promising in preventing or treating multiple diseases and age-related conditions in humans (Chaib et al. 2022). Our data clearly indicated that the metabolites of CP27 and CP36, from the crocodile gut microflora, were able to significantly inhibit NO production when compared to the positive control taxol, (which produced 100% NO, in response to stress). Consequently, metabolites of CP27 and CP36 as well as senloytic compounds significantly reduced taxol-mediated NO production at 3 h and 6 h in cerebrovascular endothelial cells. Of note, crocodile gut bacterial metabolites were even more potent at reducing NO production then the senloytic compounds, even in the presence of the positive control taxol, which further shows their protective attributes against cellular stress. This finding is noteworthy, as senloytic compounds, fisetin and quercetin, are agents that promote apoptosis of senescent cells that may accumulate with aging and at sites of pathology in multiple chronic diseases (Zhu et al. 2017). It has been shown that the reduction of the burden of senescent cells by administering senolytics, may alleviate disease connected adverse phenotypes in preclinical studies (Zhu et al. 2017). Our findings revealed that crocodile bacterial metabolites utilized herein, depicted even more protective effects that the senloytic compounds: fisetin and quercetin, and our previous studies have shown the presence of several anti-inflammatory metabolites (Siddiqui et al. 2023), thus indicative of their potential development as pre/pro/postbiotics for human health, although rigorous future studies *in vivo* will be needed to realize these expectations.

Notably, the data herein, revealed that levels of the pro-inflammatory cytokines (IL-1β, TNF-α, PGE2) were markedly reduced in the presence of metabolites of CP27 and CP36, when compared to the positive control, taxol, that induced cellular stress. Previous studies have shown that the effect of stress on the immune system leads to cytokine increase in the circulation and in the central nervous system, and in various stress models, IL-1β, appears to be an important cytokine that consistently rises following stress exposure in both blood and the brain (Golovatscka et al. 2012). Thus, our findings revealed that the crocodile gut bacterial metabolites of CP27 and CP36 were able to reduce taxol-mediated NO production, as well as the production of pro-inflammatory cytokines (IL-1β, TNF-α, PGE2), and thus may be implicated in providing neuroprotective effects, (herein human cerebral microvascular endothelial cells were chosen because they play a vital role in the microvasculature responsible for forming the BBB) against cellular stressors.

We identified the metabolites in CP27 and CP36 and the most abundant metabolites consisted of several anti-inflammatory metabolites, such as 3,4-dihydroxyphenylglycol and 5-methoxytryptophan. In addition, a number of additional metabolites, including nifedipine, 4-chlorotestosterone-17-acetate, and 3-phenoxypropionic acid, were linked to anticancer activity (Khan et al. 2021; Siddiqui et al. 2023). Furthermore, these metabolites were distinct when compared to similar bacteria, suggesting the unique properties of crocodile gut bacteria (Siddiqui et al. 2023). Other molecules identified include lactic acid, f-honaucin A, l,l-cyclo(leucylprolyl), which are known for anti-bacterial activities and 3-hydroxy-decanoic acid which has anti-fungal abilities (Khan et al. 2021). The metabolites have been associated with other gut microbiota of humans and other animals, such as L-tryptophanol (Zheng, 2020), Urocanic acid (Zheng, 2020), allantoic acid (Chalmers, 1979), Isovalerylcarnitine (Wang,

2021). These findings are in support that the model here proposed to study the gut microbiota mimics in many aspects the bacterial natural surroundings.

Similarly, to that observed in the meta-proteomic data set, the metabolomics analysis indicate that the isolated bacterial species preserve metabolic markers that can be linked to their natural habitat and rather close interaction with the host. It is worthy notice that the list of the metabolites included number of anti-inflammatory metabolites 5-methoxytryptophan {Wu, 2021 #293}, 3,4-Dihroxyphenylglycol (Fernández-Prior, 2021). Interestingly, there were several other metabolites associated with anticancer actions such as nifedipine (MotieGhader, 2022), 4-chlorotestosterone-17-acetate (Zhang, 2022), 3-phenoxypropionic acid (Neidlein, 1990). Based on these findings, it is suggested that the crocodile gut microbiota may explain in part why these animals are noted for not suffering from intestinal inflammatory disorders. Thus, the metabolites of CP27 and CP36 may be of benefit for human health, and the results from the current study are indicative of their unique potential in providing protection from cellular stress, as well as reduction of pro-inflammatory markers.

Among abundant metabolites more prevalent in CP27 there were 2-Methyl-4-nitroimidazole, N-Acetyl-L-tyrosine, Acetaminophen, Trans-Ferulic acid, N, N-Dimethylformamide, Pyrocatechol, Cyclohexanone, 3, 4-Dihydrozphenylglycol, Diphenhydramine, Melatonin, Gamma –terpinene. Whereas in CP36 samples the most abundant metabolites were Carbamazepin, deoxyninosine, Cysteamine, Benzylnicotinate, 3-phenoxypropionic acid, Indole-3-carbinol, Benzaldehyde, Benzocaine, 2-Aminobenzoic acid, 3-Methylindole. Functional enrichment analysis of all identified metabolites with metabolite sets based on drug pathways showed that they were enriched for drug action of top ten pathways associating with enalapril metabolism pathway, diphenhydramine H1-Antihistamine action, enalarpil action pathway, benzocaine action pathway, mepivacaine action pathway, oxybuprocaine action pathways, nifedipine action pathway, propranolol action pathway, acetaminophen metabolism pathway, carbamazepine metabolism pathway. When cerebrovascular endothelial cells were exposed to stress, the metabolites were more potent in inhibiting nitric oxide production than senloytic compounds (fisetin, quercetin). Stress-induced release of pro-inflammatory cytokines (human interleukin 1 beta, human tumor necrosis factor alpha, and Prostaglandin E2) by cerebrovascular endothelial cells was inhibited by the metabolites. Overall, crocodile gut bacteria, CP27 and CP36 contain metabolites to exact their effects [(3,4-dihydroxyphenylglycol, 5-methoxytryptophan, nifedipine, 4-chlorotestosterone-17-acetate, 3-phenoxypropionic acid, lactic acid, f-Honaucin A, l,l-Cyclo(leucylprolyl), 3-hydroxy-decanoic acid etc.]. Epigenetics studies using genome-wide DNA methylation analysis revealed that metabolites exhibit anti-inflammatory effects, indicative of their potential in providing protection against cellular stress. Although the present research has identified the underlying molecular mechanisms *in vitro*, future *in vivo* studies are needed to elucidate their value in the rational development of innovative therapies against cellular stress/gut dysbiosis.

Results dissemination Results have been published in 9 peer reviewed journals and also presented at 2 international conferences as described below.

Publications:

1. **Siddiqui, R.,** Akbar, N., Maciver, S. K., Alharbi, A. M., Alfahemi, H., Khan, N. A. **(2023).** Gut microbiome of *Crocodylus porosus* and cellular stress: inhibition of nitric oxide, interleukin 1-beta, tumor necrosis factor-alpha, and prostaglandin E2 in cerebrovascular endothelial cells. *Archives of Microbiology* 205: e344. doi.org/10.1007/s00203-023-03680-z
2. **Siddiqui, R.,** Akbar, N., Soares, N.C., Al-Hroub, H.M., Semreen, M.H., Maciver, S.K., Khan, N.A. **(2023).** Mass spectrometric analysis of bioactive conditioned media of

bacteria isolated from reptilian gut. *Future Science OA.* 9(5): FSO861. doi:10.2144/fsoa-2023-0030.

3. **Siddiqui, R.,** Muhammad, J. S., Maciver, S. K., Khan, N. A. **(2022).** *Crocodylus porosus* sera a potential source to identify novel epigenetic targets: *in silico* analysis. *Veterinary Sciences* 9: e210. doi.org/ 10.3390/vetsci9050210
4. **Siddiqui, R.,** Maciver, S. K., Khan, N. A. **(2022).** Gut microbiome-immune system interaction in reptiles. *Journal of Applied Microbiology* 132(4):2558-2571. doi: 10.1111/jam.15438.
5. **Siddiqui, R.,** Mungroo, M. R., Alharbi, A. M., Alfahemi, H., Khan, N. A. (2022). The use of gut microbial modulation strategies as interventional strategies for ageing. Microorganisms 10: e1869. doi: https://www.mdpi.com/2076-2607/10/9/1869
6. **Siddiqui, R.,** Soopramanien, M., Alharbi, A. M., Alfahemi, H., Khan, N. A. (2022). Novel Sources of Bioactive Molecules: Gut Microbiome of Species Routinely Exposed to Microorganisms. *Veterinary Sciences* 9: e380. Doi: https://www.mdpi.com/2306-7381/9/8/380
7. **Siddiqui, R.,** Qaisar, R., Al-Dahash, K., Altelly, A. H., Elmoselhi, A. B., Khan, N. A. **(2023).** Cardiovascular changes under the microgravity environment and the gut microbiome. *Life Sciences in Space Research*, ISSN 2214-5524, https://doi.org/10.1016/j.lssr.2023.09.003.
8. **Siddiqui, R.,** Khan, N.A. **(2023).** Microbiome and One Health: Potential of Novel Metabolites from the Gut Microbiome of Unique Species for Human Health. *Microorganisms* 11(2):481. doi: 10.3390/microorganisms11020481.
9. **Siddiqui, R.,** Elmoselhi, A.B., Khan, N.A. **(2023).** Space medicine: gut microbiome of hardy species is a potential source to counter disorders during space travel. *Future Science OA.* 9(7):FSO868. doi: 10.2144/fsoa-2023-0060.

Conferences:

I have presented my work at two conferences as follows:

1. American Society for Microbiology Annual Meeting, Houston, TX, USA (June 15 – 19, 2023). Ruqaiyyah Siddiqui, Noor Akbar, Nelson Cruz Soares, Hamza Mohammad Al-Hroub, Mohammad Harb Semreen, Sutherland K. Maciver, Naveed Ahmed Khan "Crocodylus porosus gut bacteria as a potential source of antibacterials".
2. American Society for Microbiology Annual Meeting, Houston, TX, USA (June 15 – 19, 2023). Naveed Ahmed Khan, Shama Shama, Anu V Ranade, Rizwan Qaisar, Isfahan Tauseef, Adel Elmoselhi, Ruqaiyyah Siddiqui. "Gut bacteria of crocodile are beneficial to microbial diversity as well as multi-organ health in in vivo model of microgravity using hind-limb unloaded mice".
3. 19th international Free-Living Amoebae Meeting (FLAM), France (June 26 – 30, 2023). Naveed Ahmed Khan, Noor Akbar, Alexander D Giddey, Nelson C Soares, Ruqaiyyah Siddiqui. "Gut bacteria of water monitor lizard are a potential source of antiamoebic molecule(s)".
4. 19th international Free-Living Amoebae Meeting (FLAM), France (June 26 – 30, 2023). Ruqaiyyah Siddiqui, Zinb Makhlouf, Noor Akbar, Mustafa Khamis, Taleb Ibrahim, Amir Sada Khan, Naveed Ahmed Khan. "Novel properties of deep eutectic solvents against Acanthamoeba castellanii belonging to the T4 genotype".

Impact

Impact on society beyond science and technology

This is an exciting, unique and novel research area with potential for tremendous impact on human health and performance. Our results have identified several efficacious molecules that will be of benefit for human health. The concept of using bacteria is not unusual, for example, "Mutaflor©" (specific strain of *E. coli*) is widely used as a probiotic and Captopril, a blood pressure drug was developed from snake venom. These are successful examples and the use of crocodile gut bacterial metabolites is not as far-fetched. Our findings have revealed metabolites of potential translational value to improve performance and wellbeing of humans. Although the present research has identified the underlying molecular mechanisms *in vitro*, future *in vivo* studies are needed to elucidate their value in the rational development of innovative therapies against cellular stress/gut dysbiosis.

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Technical Updates-

Please see attached 9 peer-reviewed publications during the current year of research activity, as a result of this funding.

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