Untargeted metabolomics based prediction of therapeutic potential for apigenin and chrysin

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Abstract:

Flavonoids belong to the polyphenol superfamily and present in our foods derived from natural sources including fruits and vegetables. Apigenin and Chrysin, prominent flavonoids have been demonstrated to have systemic benefits. Our previous work was first to establish the impact of apigenin and chrysin on cellular transcriptome. In the current study, we have revealed the ability of apigenin and chrysin to alter cellular metabolome based on untargeted metabolomics. Based on our metabolomics data, both these structurally related flavonoids demonstrate diverging and converging properties. Apigenin demonstrated the potential to possess anti-inflammatory and vasorelaxant properties through the upregulation of alpha-linolenic acid and linoleic acid metabolism intermediates. Chrysin on the other hand exhibited abilities to inhibit protein and pyrimidine synthesis along with downregulation of gluconeogenesis pathways based on the metabolites obtained. These metabolic effects were due to the impact of chrysin to modulate L-alanine metabolism and urea cycle. On the other hand, both the flavonoids demonstrated converging effects by their ability to downregulate metabolites involved in cholesterol biosynthesis and uric acid synthesis namely 7-dehydrocholesterol and xanthosine respectively. Our work could provide understanding regarding the various therapeutic potential of these naturally occurring flavonoids which are food constituents to treat metabolic complications in conditions like obesity, diabetes, cancer, and cardiovascular diseases. Further investigation of these natural agents for targeted diseases using whole animal and translational approaches will validate their therapeutic ability and help in curbing an array of metabolic complications.

1 . Introduction:

Foods are a direct link to our environment. Most of the global population relies on naturally available foods from plant and animal sources[1-3]. Polyphenols are naturally occurring bioactive compounds presents in vegetables, fruits and other plant parts that humans have been consuming for years[4, 5]. In recent decades, chemists have developed strategies and tools to isolate the pure individual bioactive component from a plant part or the whole plant. This is important for understanding the physiological impact of that individual component. Apigenin and Chrysin are two structurally related polyphenols belonging to flavonoid family that are present in fruits, leaves and vegetables[6, 7]. In the available literature, a lot of medical benefits associated with these are described[4-6, 8]. The rationale for using these agents in most reports published to date has been more of a "pick and choose" strategy. Most of the published work has only portrayed the beneficial effects associated with apigenin and/or chrysin in the area of cardiovascular disease, cancer, or neurodegeneration[4-6, 8]. From a pharmacological perspective, it is very crucial to remember that these natural agents are not specific ligands and could regulate an array of targets, resulting in a landscape shift in cellular signaling and function. When we consume these agents through foods, we cannot tightly control the dose. There are dose specific and dose dependent pharmacological and toxicological effects that are entirely unknown and need to be identified.

We have been consistently working on determining the benefits and risks associated with the use of these agents using omics based approaches including our current and previous works[7]. In our previous work, using a transcriptomic approach, we identified that both apigenin and chrysin were able to downregulate the cholesterol biosynthesis pathway while promoting ketogenic pathway[7]. In our current work using an untargeted metabolomics approach, we have corroborated our past findings, and have demonstrated that a penultimate metabolite in the cholesterol biosynthesis pathway is down regulated. Interestingly along with some existing similarities, we were able to observe quite diverse properties in regulating cellular metabolites by these two related polyphenols. Based on this omics approach, we will be able to predict their potential application or usage. It will reveal both their potential benefits and associated risks, which could help us in judicious use of these bioactive agents. This work will reveal a plethora of information regarding these two polyphenols and will help us in determining whether they can be selected as ideal therapeutic agents along with their suitability for specific indications.

2 . Materials and Methods:

2.1 Cell culture and treatments

Mouse Embryonic Fibroblasts (MEFs) purchased from Lonza Walkersville, Inc. (MD, USA) with Cat#M-FB-481 were employed for our experiments. Healthy passages from 2–5 was used for the different treatment groups. The cells were cultured and passaged in Dulbecco's Modification of Eagle's Medium (DMEM) (Corning®, USA). DMEM comprising high glucose (4.5 g/l) was filtered along with 10% Fetal Bovine Serum, (FBS00), 500 ml, USDA-Origin, Neuromics, MN, USA. 1.5 % penicillin/Streptomycin and amphotericin from VWR, Avantor, USA. During treatment, the serum containing medium will be replaced with a serum free 1g/L glucose containing (DMEM) medium supplied with only 1.5 % penicillin/Streptomycin and amphotericin. The cells were subjected to only serum free 1g/L glucose containing (DMEM) medium (controls) or incubated with 25 µM Apigenin in 1 g/l glucose containing DMEM or c) 25 µM Chrysin 1 g/l glucose containing DMEM for 24 h before they are pellet, and shipped at freezing temperatures using dry ice to perform untargeted metabolomics.

2.2 Sample Preparation:

The untargeted metabolomics service was performed at our outsourcing facility (Creative Proteomics, NY, USA). In brief, the shipped cell pellets were thawed, and 80-85% of methanol was added to cover the pellets (750-1000 µl). These sample sets were subjected to ultrasound-based extraction at a steady temperature set at 4°C for approximately 30 minutes. Following ultrasound exposure, samples were kept at -40°C for at least an hour. After the cold exposure, samples were removed, vortexed well for 30 seconds and then centrifuged at a speed of over 12000 rpm at 4°C for at least 12-15 minutes. A clear supernatant from the top layer of approximately 200µl and DL-O-Chloro-phenylalanine at a concentration of 140 µg/mL made into 2-5 µl was transferred to vial for LC-MS analysis.

2.3 Chemical Structures:

The chemical structures with the IUPAC names were generated using the software- ChemDraw Prime v19.1 from Perkin Elmer

2.4 UPLC-TOF-MS Technology

The Ultra Performance Liquid Chromatography (UPLC) with Time-of-flight Mass Spectrometry (ESI-TOF-MS) was performed at our outsourcing facility by a well established method[9, 10]. In brief, the sample separation was performed using Ultimate 3000LC combined with Q Exactive Mass Spectrometry (Thermo) followed by screening with ESI-MS. The LC system is a combination of two systems unit with Thermo hyper gold C18 (100×2.1mm 1.9 µm) combined with the Ultimate 3000LC system. The mobile phase comprises of 2 solvents – Solvent A and Solvent B. Solvent A comprises of 0.1% formic acid, 5% acetonitrile and water and Solvent B is a

mixture of 0.1% formic acid and acetonitrile with a gradient elution of 0-1.5 min, 0-20% B; 1.5-9.5 min, 20-100% B; 9.5-14.5 min, 100% B; 14.5-14.6 min, 100-0% B; 14.6-18.0 min, 0% B. The flow rate for the mobile phase was fixed at 0.3 ml/min. The column temperature is maintained at 40°C, and the sample manager temperature is set at 4°C. Mass spectrometry parameters in ESI+ and ESI- mode are the following. For positive ion mode (ESI+) the experimental parameters were the following: with heater temperature set at 300 °C, flow rate of the sheath gas at 45 arb, auxiliary gas flow rate at 15 arb, sweep gas flow rate at 1 arb with a spray voltage of 3.0 kV, the capillary temperature is set at 350 °C with the S-Lens RF Level adjusted to 30%.

2.5 Statistical Analysis

The analysis was performed using well established previously published statistical method[10, 11]. Following the acquisition of the raw data, will be aligned with the aid of Compound Discover using 3.0 system from Thermo based on their m/z ratio and the retention times of ion signals. The emerging ions from both the positive (ESI+) and negative (ESI-) ion modes are fused before importing into the SIMCA-P program (version 14.1) for multivariate analysis. A preliminary unsupervised method was employed for Principal Components Analysis (PCA) for visualization of data and for the identification of outliers. The data sets are then subjected to a supervised version of regression modeling using Partial Least Squares Discriminant Analysis (PLS-DA) or Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) to identify the target metabolites. The filtered out metabolites are confirmed by combining the obtained results with that of variable importance in projection (VIP) values. The VIP values > 1.5 and p value < 0.05 based on t-test were taken into consideration. The quality of data fit is then explained with the help of R2 and Q2 values. R2 indicates the variance and denotes the quality of the fit explained in the model. Q2 indicates the variance in the data with the model's predictability.

From the obtained raw values, fold change and log 2 fold change was calculated. A log 2 fold change of > +/- 1.5 with a statistical significance as indicated by $P<0.05$ was considered as a significant change. The calculated values were analyzed using GraphPad Prism software to evaluate the statistical significance of the test and difference between groups and plotted.

3 . Results:

3. 1. Apigenin and Chrysin treatment altered the whole cell metabolome in MEF cells.

The two closely related flavonoids, apigenin and chrysin (**Fig 1 A**) altered the cellular metabolome in MEFs. Metabolites obtained from both negative and positive ion mode (ESI- and ESI+) were analyzed using Principal Components Analysis (PCA), Partial Least Squares Discriminant Analysis (PLS-DA) or Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) to reveal the different metabolites formed following apigenin and chrysin treatments in comparison to control treatment. A clear distinction was evident between the cluster of formed metabolites in apigenin and chrysin treated groups in comparison to the control groups in both ESI- (**Fig 2 A – Left Upper Panel**) and ESI+ mode (**Fig 2 B – Left Lower Panel**). Volcanic plot of metabolite data sets validated the findings that apigenin and chrysin can make that clear cut distinction in the metabolite

changes. Univariate analysis of volcanic plot of metabolite sets when expressed as Log 2-fold change revealed that apigenin was able to upregulate and chrysin was able to downregulate some of the major metabolite markers within the fibroblast cells as exhibited by data sets from both negative (**Fig 2 C Right Upper**) and positive (**Fig 2 D Right Lower**) ion modes. The increase or decrease in metabolite levels as depicted by data points in volcanic plot, (colored) pink is in the significant range. These data sets reveal the ability of these closely related flavonoids in regulating cellular metabolome

3. 2. Alpha linolenic acid and linoleic acid metabolism emerged as the major metabolic pathways specifically regulated by apigenin

Pathway enrichment analysis of altered metabolites revealed that alpha linolenic and linoleic acid pathways were the prominently regulated pathways by apigenin unanimously in both positive and negative ion mode as depicted in **Fig. 3 right** and **Fig. 3 left panels** respectively. The enrichment ratio along with the P-value reached at significant levels for the metabolites are selected. Specific analysis of metabolites belonging to these two major pathways regulated by apigenin revealed eicosapentaenoic acid, docosapentaenoic acid and docosahexaenoic acid were the major ones. When comparing metabolites, the parameters were set to either> ± 1. 5-fold change(increase/decrease) as per the *Log 2-fold change* values with a statistical significance set at *P<0.05.* Eicosapentaenoic acid levels in apigenin treated MEFs showed a significant increase with log 2 fold change values **>1.7** fold as compared to control groups and even chrysin treated groups based on the values from negative ion mode (**Fig. 4 A**). Docosapentaenoic acid and Docosahexaenoic acid exhibited only > 1.3 and >1.04 respectively as per the log 2-fold change expressed in negative ion mode in comparison to both control and chrysin treatment groups (**Fig . 4 B and Fig. 4 C**) and didn't fulfill the selection criteria. For metabolites in the linoleic acid pathway, the metabolites that appeared in the list were arachidonic acid and adrenic acid. The changes in arachidonic levels were statistically significant (P<0.05) but the log 2-fold change didn't reach up to 1.5-fold rather it was 0.671 fold increase (**Fig.5 A**). Interestingly, adrenic acid reached over > 2.88 log 2-fold change (increase) along with the statistical significance (P<0.05) compared to both control and chrysin treated groups (**Fig.5 B**). The original peak values for the metabolites are available as upper inserts in these figures.

3. 3 Alanine metabolism and urea cycle are the major metabolic pathways specifically controlled by Chrysin

The metabolite sets which emerged as the top regulated ones by chrysin in positive (**Fig. 6 right panel**) and negative mode (**Fig. 6 left panel**) as revealed by pathway enrichment analysis were urea cycle and alanine metabolism respectively. L- Alanine metabolism is tissue specifically regulated. In non hepatic cells like in MEFs, L-alanine can be formed from pyruvate via glucose or lactate sources (**Fig. 7 A**). The formed L-alanine can be either released into the circulation, which can be taken up by liver for the process of gluconeogenesis or can be used for the synthesis of

proteins. It can also serve as an amino acid source for ATP generation in normal tissues and also in cancerous tissues. When measured, L-Alanine levels decreased > 1.7 fold (-1.78) as expressed by the log 2 fold change with the statistical significance in comparison to control and apigenin treated MEFs (**Fig . 7 B**). We also analyzed the levels of pyruvate and lactate. Pyruvate expressed in protonated form as pyruvic acid didn't reach the cut off criteria (< or > 1.5 fold), rather only demonstrated a 0.815 fold decrease (**Fig . 7 C**). Unlike pyruvic acid, lactic acid levels demonstrated >2.09 fold decrease (-2.09) as expressed by log 2 fold change levels in comparison to control and apigenin treated groups (**Fig . 7 D**). The levels of L-alanine, pyruvic acid and lactic acid correlated well demonstrating the channelization of this metabolic pathway. Regarding urea cycle, the intermediates within the urea cycle; L-arginine and D-ornithine did not exhibit major change compared to control or apigenin treated groups (**Fig . 8 B and C**). A major precursor or substrate provider for urea cycle is carbamoyl phosphate, which has 2 major fates- either help in formation of citrulline or can take an alternative path forming Orotidine as shown in **Fig . 8 A**. Carbamoyl phosphate is formed from N-acetyl glutamate in the presence of carbon dioxide, ammonium ion and ATP. N-acetyl glutamate is formed by the combination of glutamate and acetyl coA (**Fig . 8 A**). In the absence of any change in the metabolites/substrates of urea cycle, we measured the levels of orotidine, N-acetyl glutamic acid and glutamic acid, the protonated forms of N-acetyl glutamate and glutamate were the ones appeared in the metabolite list. Interestingly, there was over 2.457-fold level decrease in orotidine (***>-2.457 fold,*** **Fig . 8 D**) which correlated well with over 1.52 decrease ***(>-1.52 fold,*** **Fig . 8 E**) for N-acetyl glutamine and over 2.30 fold decrease ***(>-2.30 fold,*** **Fig . 8 F**) for L-glutamic acid, which are the upstream metabolites of orotidine as expressed by log 2-fold change values. Orotidine is a major precursor for pyrimidine synthesis and is known to generate pyrimidine bases. In our metabolite list the downstream metabolite of orotidine and a pyrimidine base; uracil demonstrated a significant change with log 2 fold change over -2.675 change (Supplementary Figure-3). The nucleotide form of uracil and cytosine; uridine monophosphate and cytidine monophosphate also demonstrated significant changes with their log 2 fold change values over > -2.17 and >-1.89 respectively (decrease) (Supplementary Figure - 4).

The peak/raw values for the metabolites belonging to different groups are represented as upper inserts in each figure.

Apigenin and Chrysin demonstrated similarity in downregulating metabolites involved in cholesterol and uric acid biosynthesis pathways.

Along with the divergent properties demonstrated by both flavonoids- apigenin and chrysin in regulating specific metabolites, they both also exhibited some commonality. Major metabolites that were regulated in a similar manner by both apigenin and chrysin, were 7-dehydrocholesterol and xanthosine. Both 7-dehydrocholesterol and xanthosine are the major intermediate substrates/metabolites in cholesterol and uric acid biosynthesis pathways. 7-dehydrocholesterol demonstrated over 1.618 fold ***(>-1.618***) and 2.605 fold ***(>-2.605***) decrease as expressed by the log 2 fold change values for apigenin and chrysin respectively (**Fig . 9 A**). Xanthosine

demonstrated a decrease of over 2.23 fold ***(>-2.23***) and over 5.963 fold ***(>-5.963***) for apigenin and chrysin respectively as expressed by the log 2 fold change values (**Fig . 9 B**). The original peak values for the metabolites are expressed as upper inserts inside the figures.

4 . Discussion:

There is not much known about the impact of apigenin and chrysin on the cellular metabolome. Rather than directly advocating for the natural compounds of interest in treating a specific disease, it is important to understand in depth the overall impact of using these flavonoids at cellular level. Our previous work demonstrated the influence of apigenin and chrysin at the cellular transcriptome level[7]. Current work has furthered the information by revealing the impact these compounds have on cellular metabolome. Along with their ability to turn on or off certain transcripts as demonstrated by our previous work, this study with the application of untargeted metabolomics revealed their ability to change the metabolite levels inside the cell. The data has revealed an in-depth knowledge regarding how these compounds could influence cellular function by altering various metabolic pathways. Also, it is crucial to consider the pros and cons for these agents before we even advocate them for specific condition. Apigenin and Chrysin are structurally similar and related flavonoids but differ in chemical structure by having one 4-hydroxyl group in the 2-phenyl for apigenin compared to chrysin as described in **Fig.1**.

Even with a difference of just one hydroxyl group **(Fig. 1),** there is a major divergence in the metabolic properties possessed and the metabolites regulated by these two compounds **(Supplementary Fig -2)**. Interestingly, in both negative and positive ion mode metabolite detection, alpha linolenic acid and linoleic acid metabolic pathways are the crucial ones regulated by apigenin in a significant manner with high enrichment ratio **(Fig. 3)**. This was a notable observation not only against the control group but also against the chrysin treated groups **(Supplemental Figure-2)**. Based on these observations we could interpret that apigenin could be an ideal agent for activating alpha linolenic acid and linoleic acid pathways. The protective role of alpha linolenic acid and linoleic acid in cardiovascular diseases and neurodegenerative diseases is well established but we have rarely perceived agents that can channelize the intracellular lipid metabolism[12-20]. Among the metabolites regulated in these pathways, EPA, DPA and DHA demonstrated changes but only EPA levels were significant and more than >1.5 fold change as expressed by Log 2 FC values. EPA levels were increased >1.7 fold whereas DPA and DHA levels were only > 1.3 and >1.04 fold respectively. It is a clinically proven fact based on data from numerous studies that prolonged DHA increase could enhance the LDL cholesterol levels in subjects, which is considered the bad cholesterol[21, 22]. In contrary, EPA provision is known to decrease the total cholesterol and triglyceride concentrations and improve cardiovascular health. In recent years, the only EPA containing FDA approved drug (Vascepa) has demonstrated incredible cardiovascular benefits that reaffirms this claim[23, 24]. Uniqueness about our findings is that apigenin can trigger the biochemical pathways involving alpha-linolenic acid pathway leading to more of EPA than any other metabolites, which could be protective not only for

cardiovascular system but also for systemic health. The other top regulated metabolic pathway by apigenin is the linoleic acid pathway as per our findings. In the pathway, both arachidonic acid and adrenic acid were upregulated, but adrenic acid was the only metabolite that demonstrated a Log 2 FC of over > 1.5 (>2.88) in comparison to arachidonic acid, which also changed > 0.671 (Log 2 FC). Adrenic acid is also known as 7,10,13,16-docosatetraenoic acid which is an omega (ώ)-6 polyunsaturated fatty acid[25]. The protective roles for adrenic acid include endothelial derived relaxation factor as demonstrated in bovine coronary artery model and adrenal cortical arteries and as an anti-inflammatory agent inhibiting leukotriene synthesis (LTB4) in neutrophils using murine model of peritonitis and arthritis[25-27]. Apigenin by enhancing endogenous adrenic levels without significantly elevating arachidonic acid could potentially enhance vasorelaxant and anti-inflammatory effects in cardiovascular and other systems but it needs further testing in vivo. These findings for the first time provide clear insight about the multiple protective signaling networks turned on by apigenin mediated major metabolites EPA and adrenic acid.

With regard to Chrysin, L-alanine metabolism was the most regulated pathway in negative ion mode and urea cycle in positive ion mode. Hepatic tissue assimilates alanine secreted by other tissues into circulation and convert them into glucose through the process of gluconeogenesis and make use of excess alanine as the raw material for the synthesis of proteins[28-30]. When there is an upregulation of L-alanine metabolism, it could potentially lead to enhanced gluconeogenesis as seen during insulin resistance, cancer[29, 31] and diabetes[31-33]. Also, excess protein biosynthesis is another hallmark of metabolic syndrome and cancer[32, 33]. By curbing L-alanine provision to liver, we could limit the upregulation of gluconeogenesis and the trigger to synthesis excess proteins by the liver therefore reducing hepatic stress. These phenomena (excess gluconeogenesis and excess protein synthesis) are very prevalent during insulin resistance, diabetes, and cancer. By curbing these pathways, we could potentially limit metabolic complications associated with diabetes and cancer. Besides L-alanine, the other top major metabolic pathway regulated by Chrysin in positive ion mode was urea cycle. Interestingly as mentioned in our results section, no significant changes were noted in the levels of intermediate metabolites in urea cycle which came up in our analyte list; Arginine and Ornithine. An alternative fate for carbamoyl phosphate, the precursor for intermediates in urea cycle is to form Orotidine, which is a known precursor for pyrimidine nucleotides[34, 35]. Most malignancies have been associated with excess pyrimidine nucleotide synthesis and inhibiting this biochemical process has been considered a major strategy to combat malignancies[36-38]. Both orotidine and its predecessor; N-acetyl glutamate has been demonstrated to be downregulated by chrysin treatment not only in comparison to control but even apigenin treated groups. We also evaluated the levels of downstream metabolites of orotidine, the pyrimidine base; Uracil **(Supplementary Fig-3)** and also its nucleotide form Uridine monophosphate along with another pyrimidine nucleotide analog cytidine monophosphate **(Supplementary Fig – 4)**. These downstream effects confirm chrysin's ability to regulate pyrimidine biosynthesis. The unique ability of chrysin to downregulate Orotidine, a pyrimidine precursor demarcates it from its flavonoid counterpart in the treatment of cancer and associated complications. By influencing both L-alanine and

orotidine mediated pyrimidine nucleotide synthesis pathways, Chrysin emerges as an ideal candidate that could curb gluconeogenesis, and preventing excess protein and pyrimidine synthesis as seen with cancer[32, 37, 38].

Even with the existing divergence, we have observed some converging features in these structurally related flavonoids. Our previous work, based on transcriptomic analysis was the first to show that both apigenin and chrysin possess hypocholesterolemic property by downregulating multiple enzymes in the mevalonate pathway[7]. In the current work, we observed consolidating evidence that both apigenin and chrysin was able to down regulate 7-dehydrocholesterol, the penultimate metabolite in the cholesterol biosynthesis pathway. Based on the observed results chrysin was able to downregulate in a robust manner even compared to apigenin (-2.6 vs -1.6). Interestingly, another novel target which was downregulated by both apigenin and chrysin was xanthosine (-2.23 and -5.9) in comparison to control. Both cholesterol and uric acid accumulation has been reported to be an initiator of metabolic complications in cardiovascular pathologies and cancer[39-41]. Uric acid is also the biochemical end product of purine metabolism, that gets elevated during both cancer and cardiovascular complications[42, 43]. Agents to lower hypocholesterolemia has been considered a major therapeutic strategy for treating cardiovascular complications and has been studied for over a decade[44, 45]. Recently NIH funded clinical trials - Colchicine Cardiovascular Outcomes Trial (COLCOT) and Low-Dose Colchicine (LoDoCo) have brought to light the significance of inhibiting uric acid using colchicine following cardiac ischemic conditions[46, 47]. Apigenin and Chrysin with their ability to simultaneously inhibit cholesterol and uric acid could serve as ideal agents to curb metabolic complications found during cancer and cardiovascular diseases.

5 . Conclusion:

To conclude, our work based on an untargeted metabolomics approach reveals the unique properties of two closely related flavonoids. Currently we don't have much information on how these agents act at cellular level dissecting their effects on the cellular metabolome. This work will lay the foundation for future studies involving apigenin and chrysin in understanding the pharmacological properties and specifically influence of these agents on the cellular metabolic landscape. As far as the **limitations** of this study are concerned, our predictions are based on untargeted metabolomics from an in vitro model system and the predictions on the systemic effects are based on extrapolations. We recommend that this work should be definitely followed up with studies involving in vivo model systems to identify the correct dose and toxicity when treating metabolic complications associated with cardiovascular disease or cancer. Both apigenin

and chrysin has demonstrated high potential to emerge as therapeutic agents that can help in curbing metabolic diseases with feasibility and with predictable and minimal adverse effects.

Author contributions

CC and KM conducted experiments and edited manuscript, DR conducted experimented and edited the manuscript. JC, AS, and AL performed experiments. SK contributed to the figures and edited the manuscript. PP conceived hypothesis, conducted experiments and collected data, wrote and edited the manuscript and was primarily involved in plotting the figures and making diagrams. All the authors have read and approved the manuscript.

Conflicts of interest

All authors declare no conflict of interest.

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Figure legends:

Figure -1: IUPAC nomenclature for structurally related flavonoids. Apigenin is a 5,7-Dihydroxy-2-(4'-hydroxyphenyl)-4*H*-chromen-4-one and Chrysin is a 5,7-Dihydroxy-2-phenyl-4*H*-chromen-4-one.

Figure-2: Metabolic profiling of mouse embryonic fibroblasts following apigenin and chrysin treatment for 24 hours. Scatter plots following Principal Components Analysis (PCA), Partial Least Squares Discriminant Analysis (PLS-DA) and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) in both negative ion mode (ESI- (2 A – Left Upper Panel)) and positive ion mode (ESI+ mode (2 B – Left Lower Panel)). The volcanic plot based metabolite profiling under negative and positive ion mode can be found on the upper right side (2 C) and lower right side (2 D) respectively.

Figure – 3: Pathway enrichment analysis of altered metabolites in mouse embryonic fibroblasts following apigenin treatment. Following 24 hours of apigenin treatment, there were numerous metabolite pathways which got altered following both negative ion mode (Fig 3 left) and positive ion mode (Fig 3 right) panels respectively.

Figure – 4: Analysis of components of alpha-linolenic acid pathway regulated by apigenin. The metabolites belonging to the alpha-linolenic acid pathway that appeared in the analysis panel were A) Eicosapentaenoic acid B) Docosapentaenoic acid and C) Docosahexaenoic acid. The raw values are plotted on the upper inserts and the raw values converted to Log 2 fold change and plotted using graph pad prism were used in the main figure. Significance was determined based on $P<0.05$ and with log 2-fold change difference of +/_ 1.5 fold change.

Figure – 5: Metabolites of linoleic acid pathway regulated by apigenin. In the linoleic acid pathway, the metabolites which were differentially regulated by apigenin were A) arachidonic acid and B) adrenic acid. The upper inserts represent the raw values. The Log 2 fold change values were plotted using graph pad and significance set at $P<0.05$ with a +/_ 1.5 fold change.

Figure -6: Metabolic pathways regulated by chrysin Pathway enrichment analysis. Both the negative ion mode (5 left) and positive ion mode (5 right) panels with their respective enrichment ratio are presented.

Figure – 7: Regulation of Alanine metabolism by Chrysin: The schematics for alanine regulation in non hepatic cells are represented A) the source of alanine is from either of the glucose metabolites- pyruvate or lactate. The formed alanine is then effluxed of the cell. B) Represents L-alanine raw values (upper insert) and log 2 fold change values. The log 2 fold change values for pyruvic acid and lactic acid which are the acidic forms for pyruvate and lactate with their respective raw values (inserts) are represented in figure 3 panel C) and D) respectively.

Figure – 8: Chrysin mediated regulation of metabolites in Urea cycle: Based on the metabolite panel urea cycle was the other top regulated metabolic pathway. A) Glutamate and acetyl CoA act as major substrates for the formation of N-acetyl glutamate and finally carbamoyl phosphate which is the precursor for substrates in the urea cycle including citrulline, arginine and ornithine. The raw values (upper inserts) and the log 2 fold change values of B) L-Arginine, C) D-Ornithine and D) Orotidine are expressed respectively. The protonated forms of N- acetylated glutamate and glutamate; N-acetyl L -glutamic acid and L- glutamic acid with the raw values (upper inserts) and calculated log 2 fold change values are represented in E) and F) respectively.

Figure – 9: Down regulation of cholesterol and uric acid pathways by apigenin and chrysin: Both apigenin and chrysin demonstrated to have a suppressive effect on cholesterol biosynthetic pathway and uric acid pathway based on the major metabolites belonging to these pathways; A) 7-dehydrocholesterol and B) Xanthosine levels as determined by raw values (upper inserts) and log 2 fold change values as expressed in respective figures.

Figure – 10: Summary diagram demonstrating the distinct and similar metabolic pathways regulated by apigenin and chrysin. Apigenin through the regulation of alpha-linolenic and linoleic acid pathways generate endogenous anti-inflammatory and vasorelaxant metabolites. On the other hand, chrysin by regulating alanine and urea cycles suppresses gluconeogenesis, protein synthesis and pyrimidine synthesis pathways. Both apigenin and chrysin demonstrates converging effect by inhibiting the generation of metabolites involved in biosynthesis of cholesterol and uric acid pathways.

Supplementary Figure 1: Metabolites differentially regulated by apigenin and chrysin. Pathway enrichment analysis and volcanic plots provide cluster of metabolites differentially regulated by apigenin and chrysin in positive (left) and negative (right) ion modes.

Supplementary Figure 2: Dot plot of enrichment analysis of perturbed metabolites in apigenin versus chrysin treated groups. The dot plot of enrichment analysis of metabolites which were altered in apigenin versus chrysin treated groups in positive ion mode (right) and negative ion mode (left) are depicted in this figure. The altered metabolites with the enrichment ratio are depicted in this figure.

Supplementary Figure 3: Decrease in orotidine mediated pyrimidine synthesis by chrysin. Along with orotidine, we also observed a chrysin mediated decrease in uracil synthesis (pyrimidine base). The raw values are presented as upper insert and log 2 fold change values are provided in the figure.

Supplementary Figure 4: Decrease in monophosphates of cytidine and uridine with chrysin. A) Orotidine usually gets converted into cytidine and uridine (pyrimidine bases) and the phosphate forms of these nucleotide bases were evaluated from the metabolite panel. The log 2 fold change values and the raw values (upper inserts) for cytidine monophosphate and uridine monophosphate were represented in B) and C) respectively.

Figures:

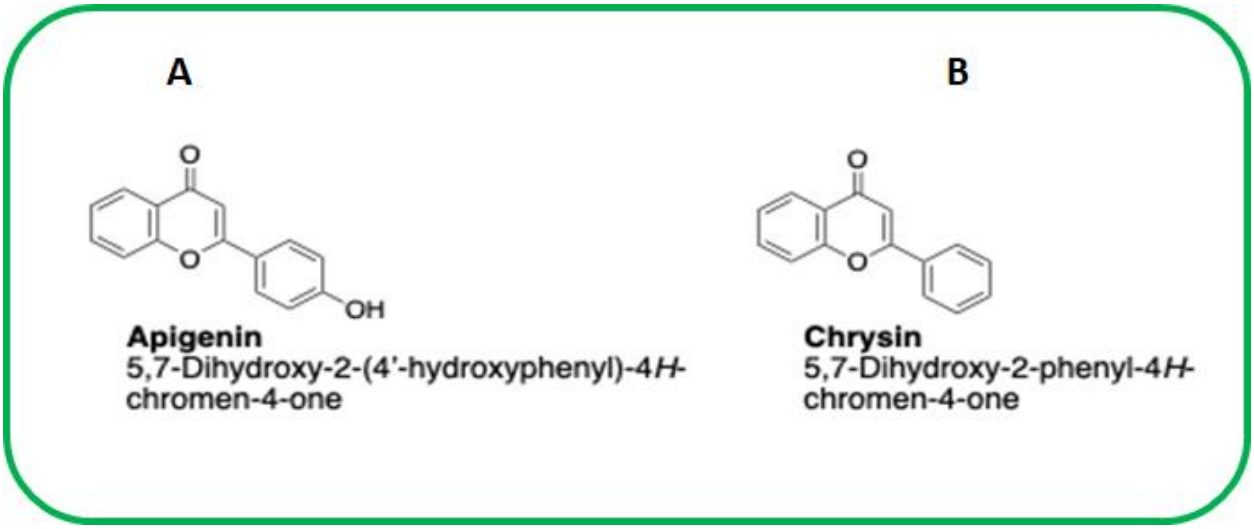


Figure -1

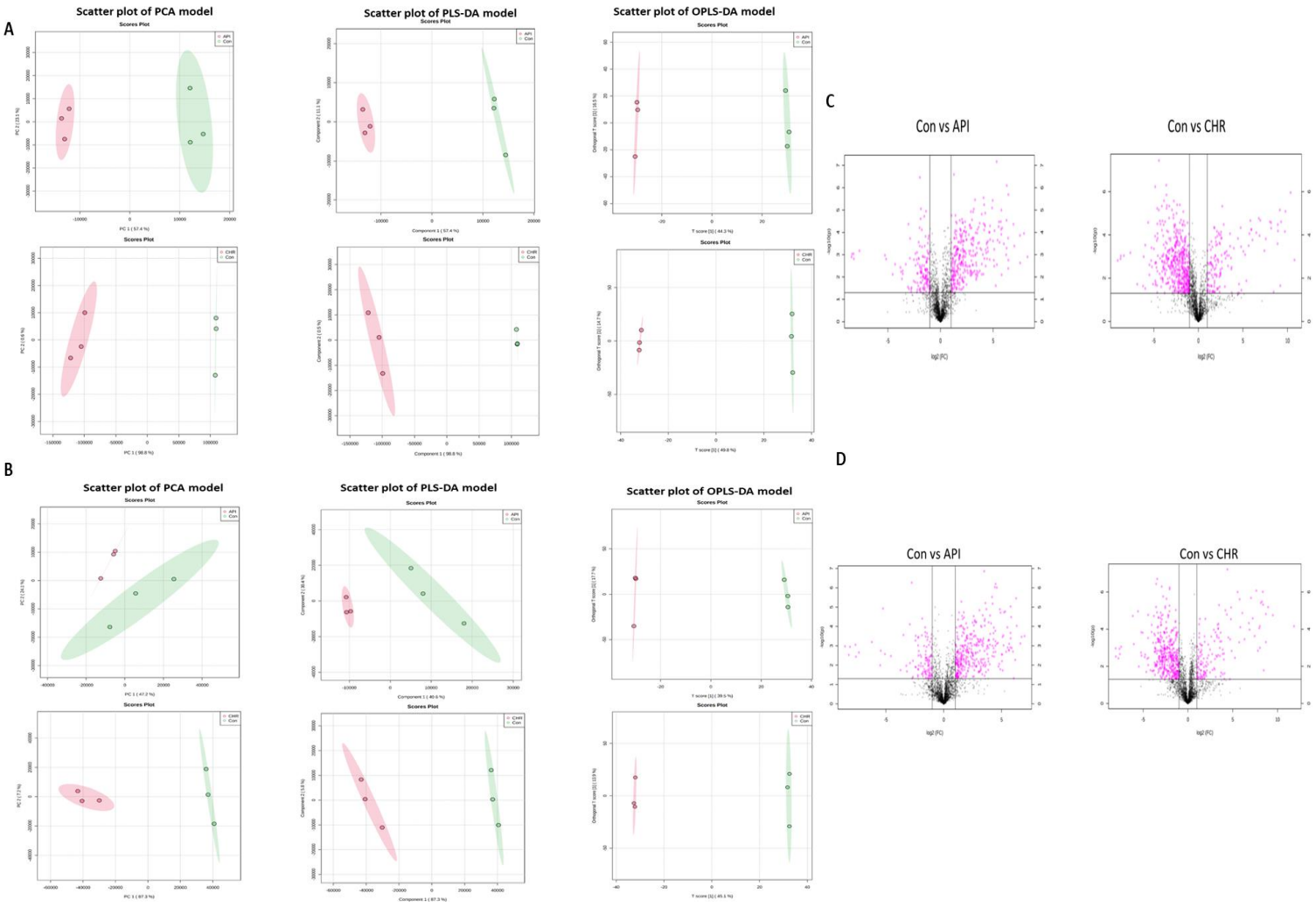
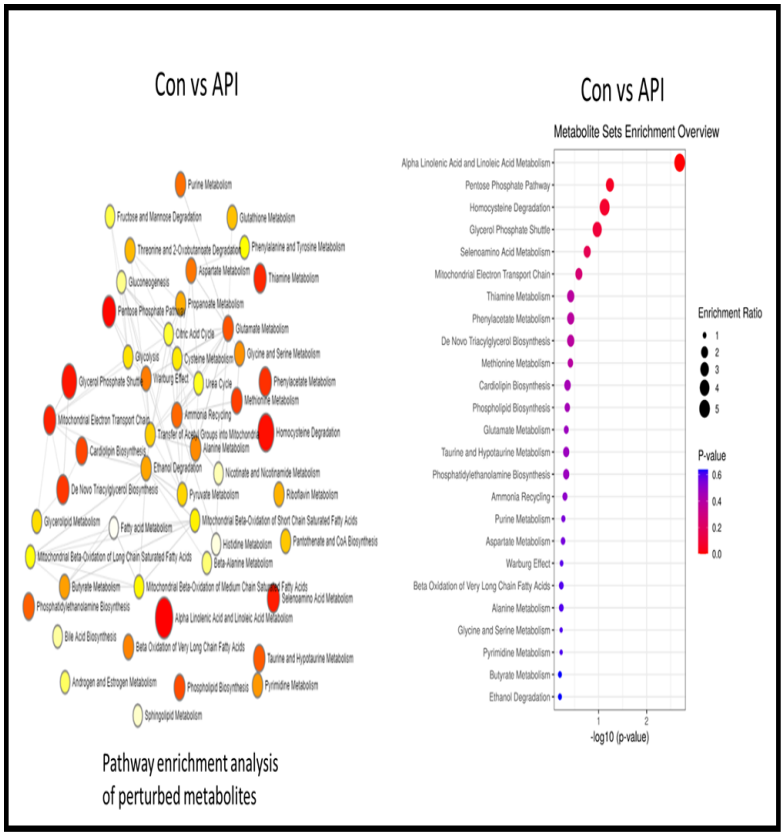
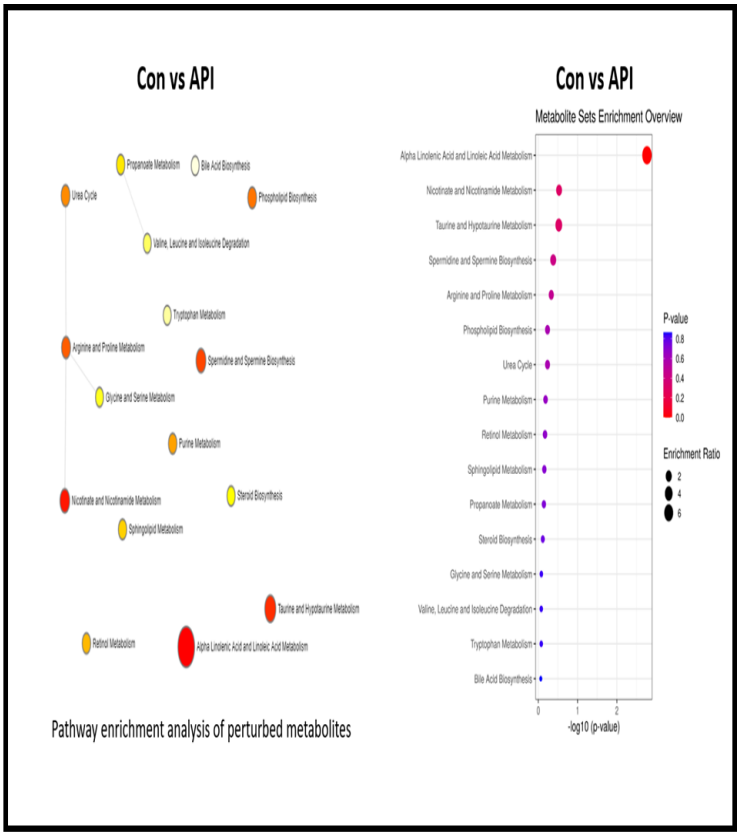

A
Scatter plot of PCA model
Scatter plot of PLS-DA model
Scatter plot of OPLS-DA model
B
Scatter plot of PCA model
Scatter plot of PLS-DA model
Scatter plot of OPLS-DA model
C
Con vs API
Con vs CHR
D
Con vs API
Con vs CHR

Figure – 2



Negative mode [ESI -]



Positive mode [ESI +]

Figure – 3

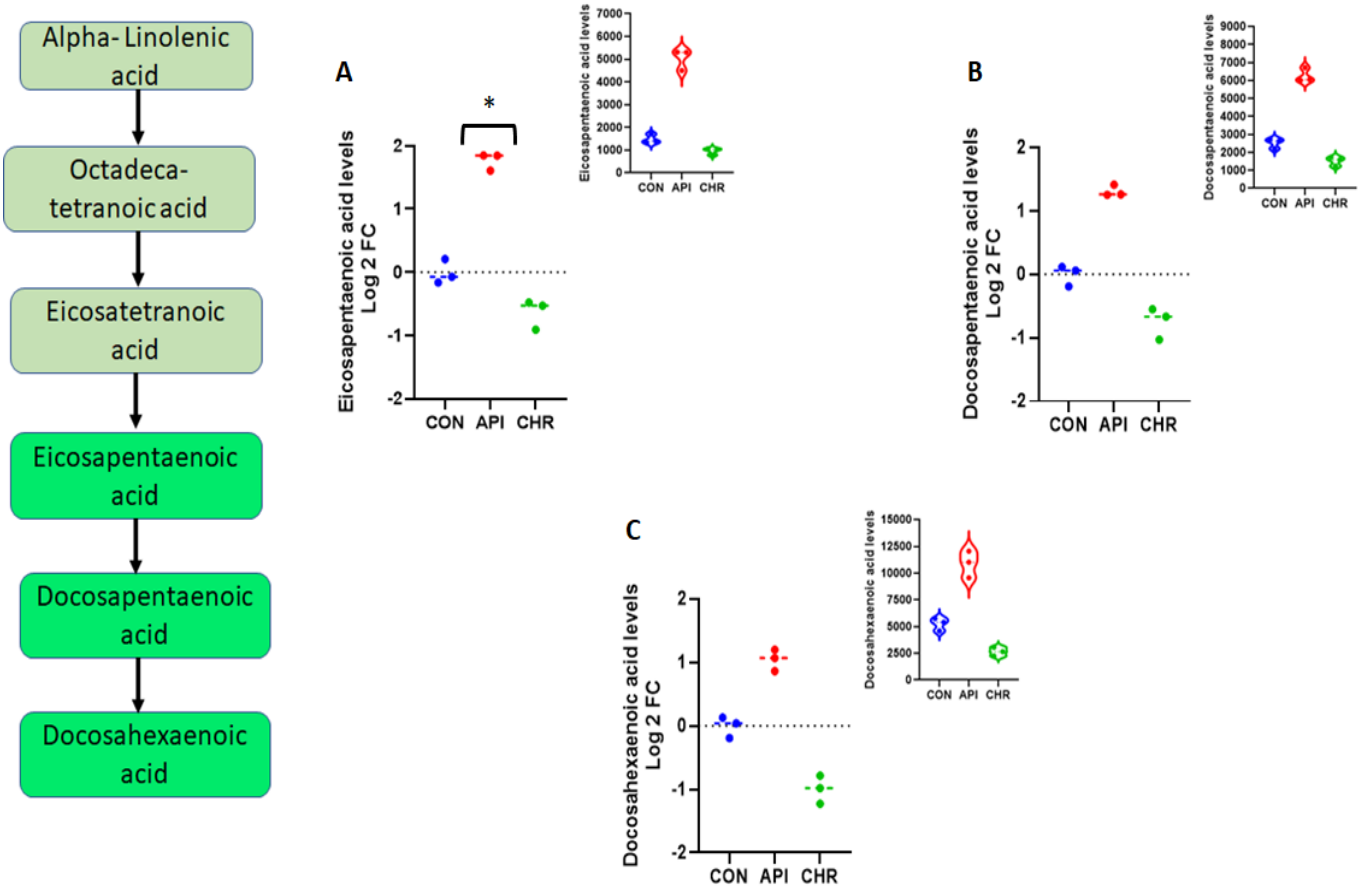

Alpha- Linolenic acid
Octadeca-tetranoic acid
Eicosatetranoic acid
Eicosapentaenoic acid
Docosapentaenoic acid
Docosahexaenoic acid
A
Eicosapentaenoic acid levels Log 2 FC
CON API CHR
Eicosapentaenoic acid levels
B
Docosapentaenoic acid levels Log 2 FC
Docosapentaenoic acid levels
C
Docosahexaenoic acid levels Log 2 FC
Docosahexaenoic acid levels

Figure – 4

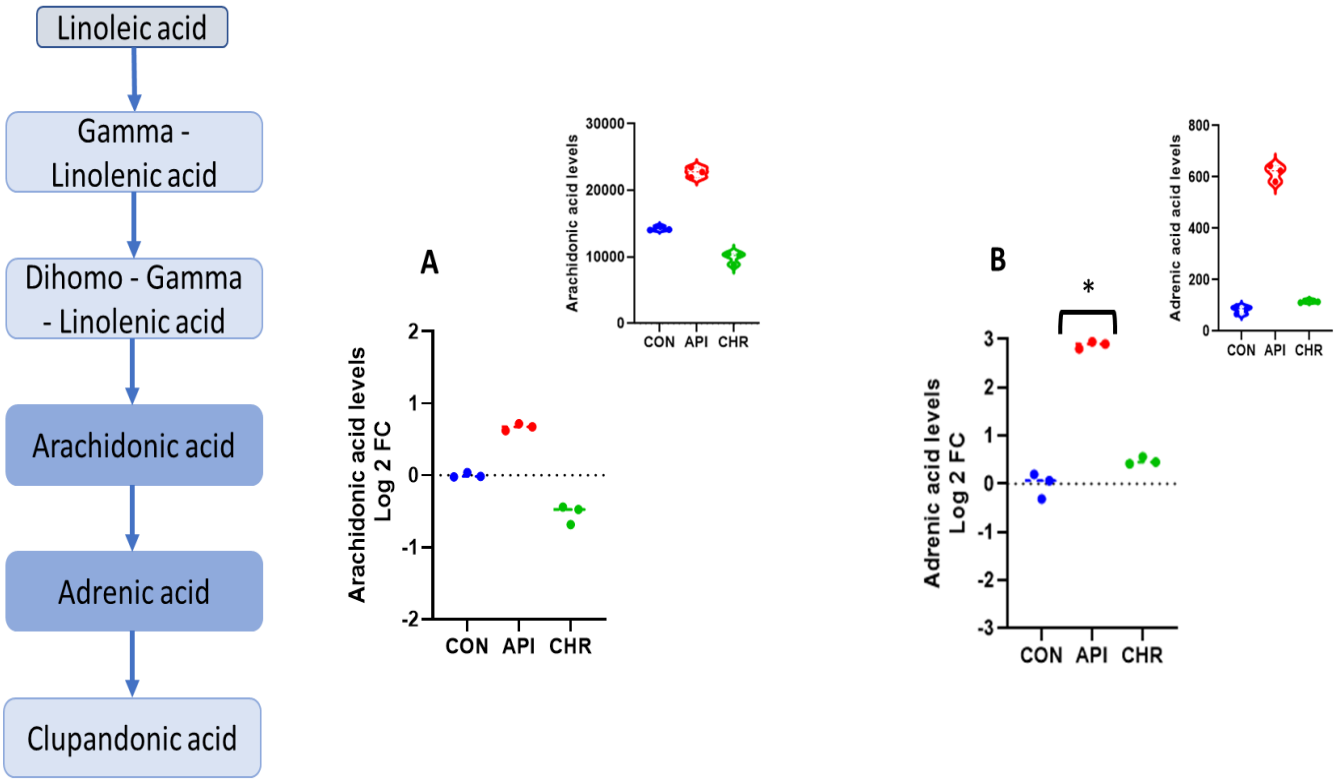
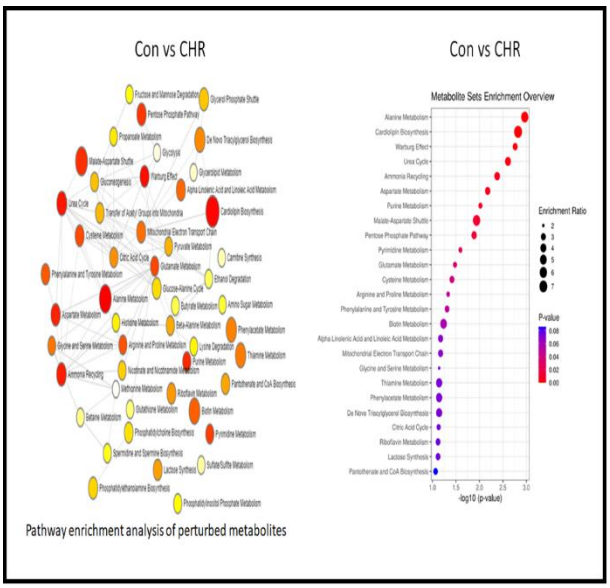
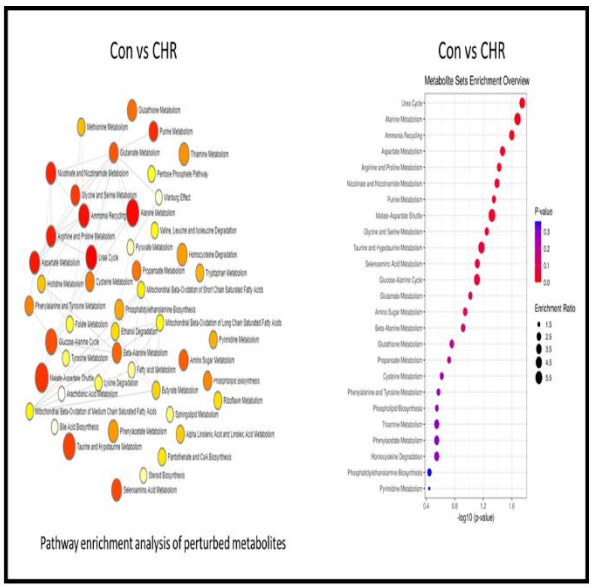

Linoleic acid
Gamma - Linolenic acid
Dihomo - Gamma - Linolenic acid
Arachidonic acid
Adrenic acid
Clupandonic acid
A
Arachidonic acid levels
Log 2 FC
2
1
0
-1
-2
CON API CHR
Arachidonic acid levels
30000
20000
10000
0
CON API CHR
B
*
Adrenic acid levels
Log 2 FC
3
2
1
0
-1
-2
-3
CON API CHR
Adrenic acid levels
800
600
400
200
0
CON API CHR

Figure – 5



Negative mode [ESI -]



Positive mode [ESI +]

Figure – 6

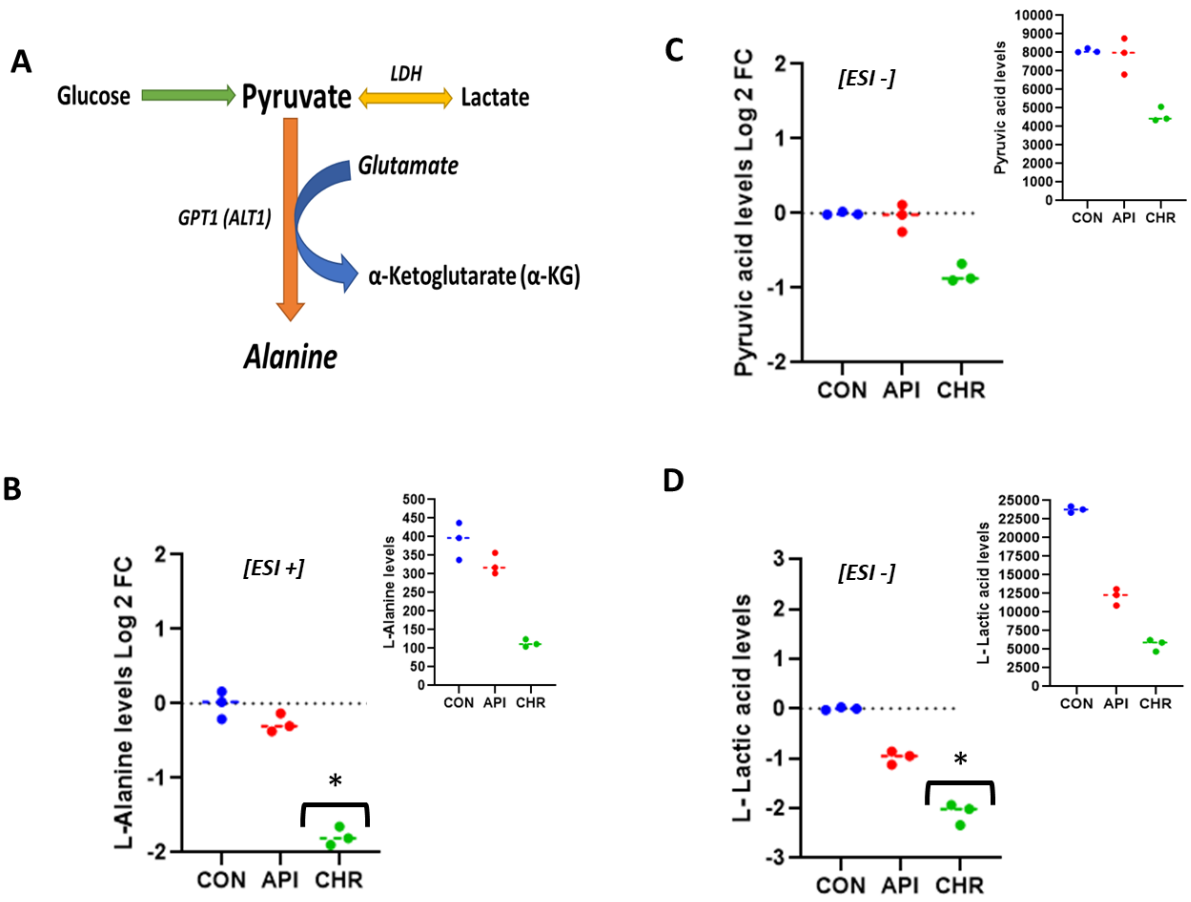

A
Glucose
Pyruvate
LDH
Lactate
Glutamate
GPT1 (ALT1)
α-Ketoglutarate (α-KG)
Alanine
B
[ESI +]
L-Alanine levels Log 2 FC
L-Alanine levels
CON API CHR
C
[ESI -]
Pyruvic acid levels Log 2 FC
Pyruvic acid levels
CON API CHR
D
[ESI -]
L-Lactic acid levels
L-Lactic acid levels
CON API CHR

Figure – 7

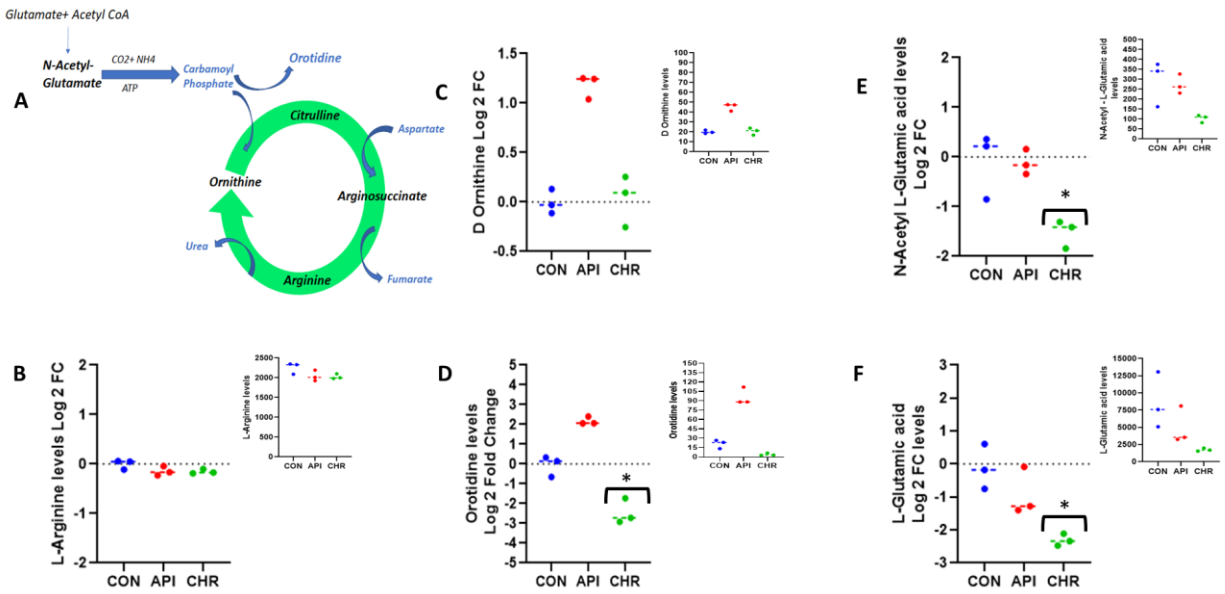

A
Glutamate+ Acetyl CoA
N-Acetyl-Glutamate
CO2+ NH4
ATP
Carbamoyl Phosphate
Orotidine
Citrulline
Aspartate
Ornithine
Arginosuccinate
Urea
Arginine
Fumarate
B
L-Arginine levels Log 2 FC
CON API CHR
C
D Ornithine Log 2 FC
D Ornithine levels
D
Orotidine levels Log 2 Fold Change
Orotidine levels
E
N-Acetyl L-Glutamic acid levels Log 2 FC
N-Acetyl - L-Glutamic acid levels
F
L-Glutamic acid Log 2 FC levels
L-Glutamic acid levels
*

Figure – 8

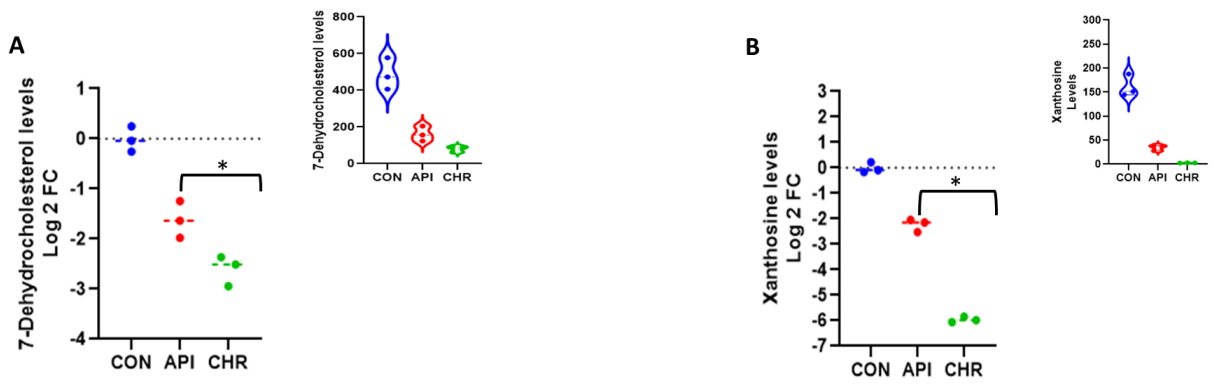

A
7-Dehydrocholesterol levels
Log 2 FC
1
0
-1
-2
-3
-4
*
CON API CHR
7-Dehydrocholesterol levels
800
600
400
200
0
CON API CHR
B
Xanthosine levels
Log 2 FC
3
2
1
0
-1
-2
-3
-4
-5
-6
-7
*
CON API CHR
Xanthosine Levels
300
250
200
150
100
50
0
CON API CHR

Figure – 9

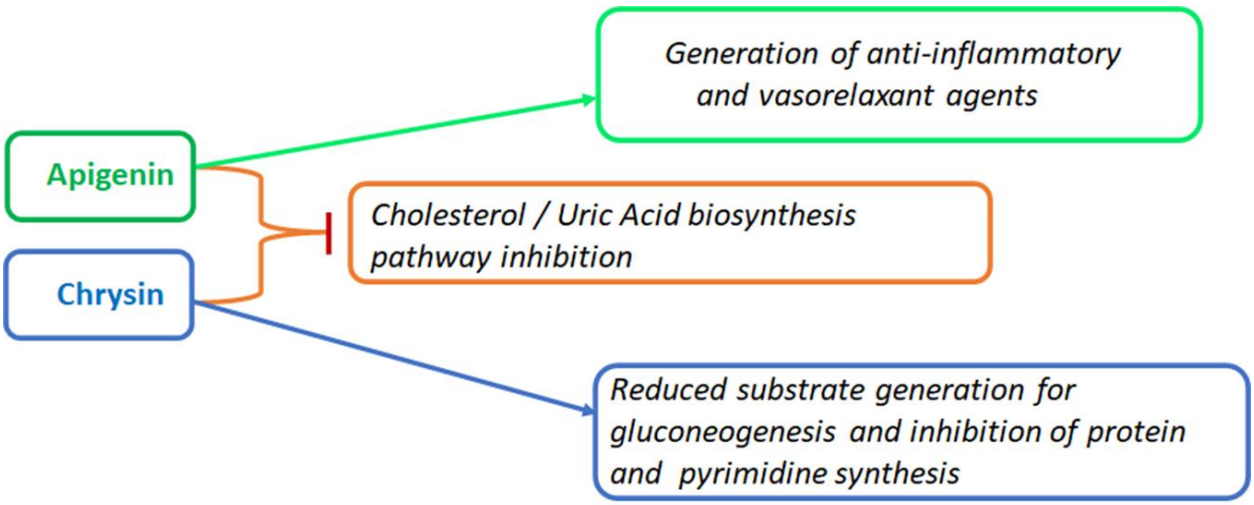
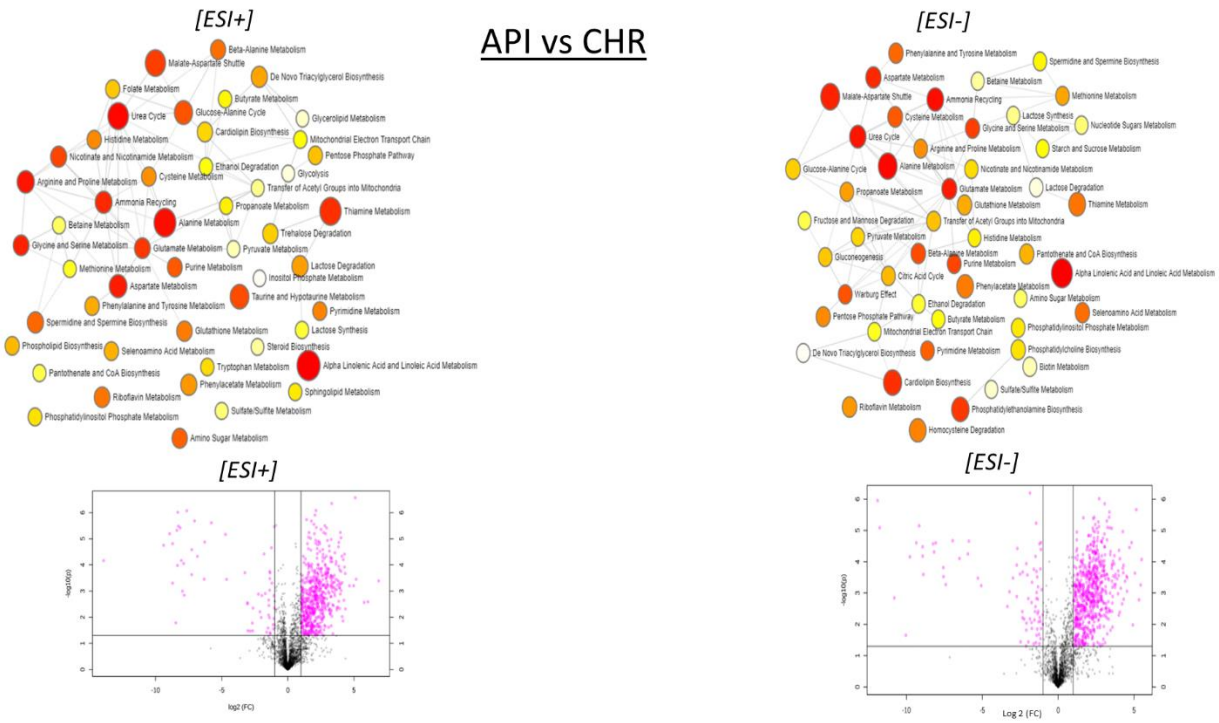

Generation of anti-inflammatory and vasorelaxant agents
Apigenin
Cholesterol / Uric Acid biosynthesis pathway inhibition
Chrysin
Reduced substrate generation for gluconeogenesis and inhibition of protein and pyrimidine synthesis

Figure – 10



Supplementary Figure -1

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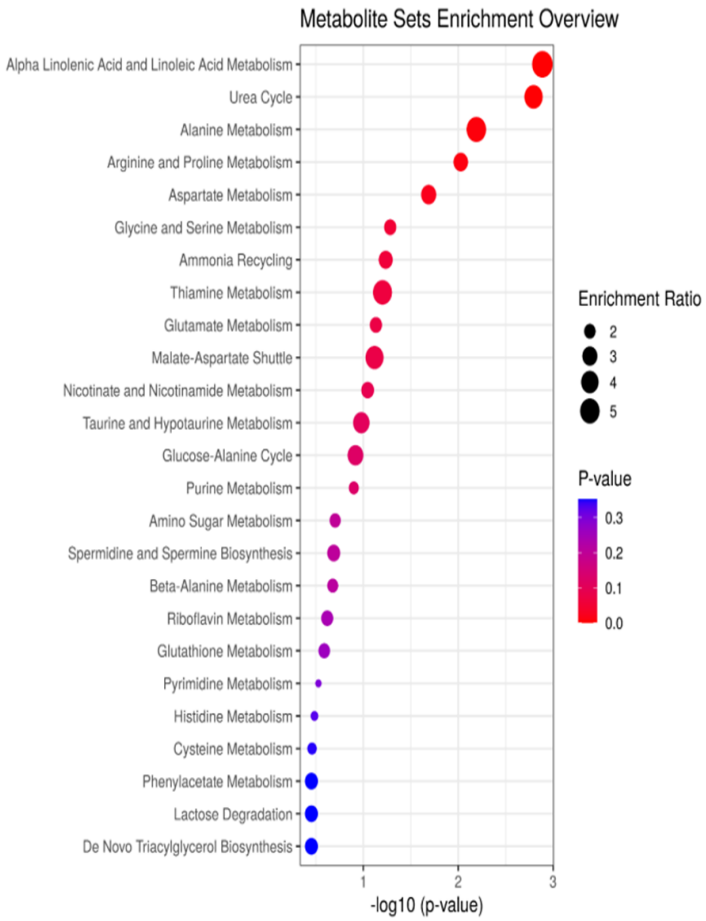
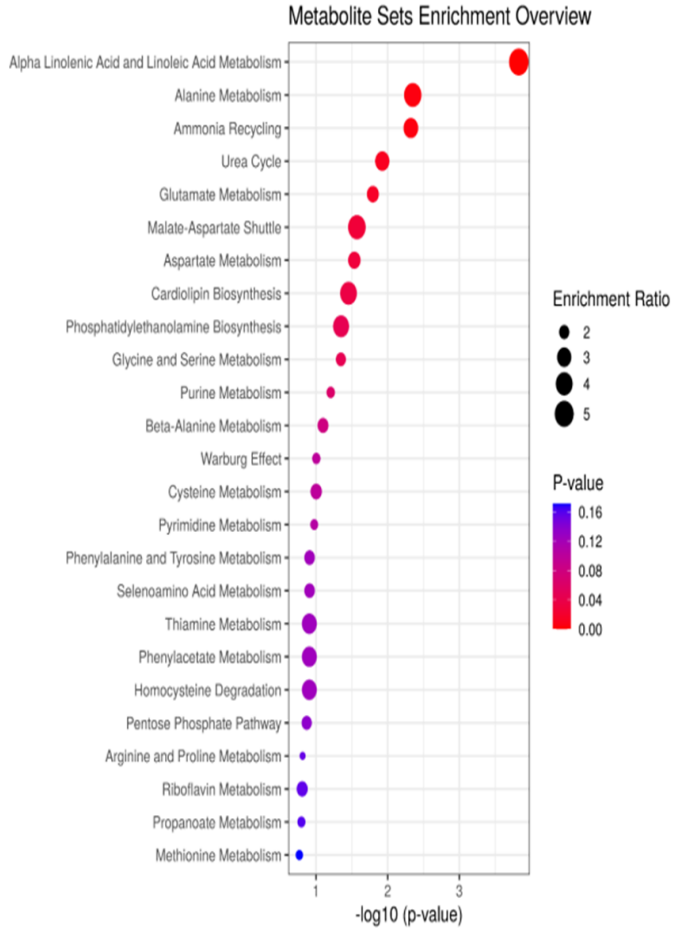
API vs CHR

[ESI+]



Dot plot of enrichment analysis in perturbed metabolites

[ESI-]



Dot plot of enrichment analysis in perturbed metabolites

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681 **Supplementary figure – 2**

682

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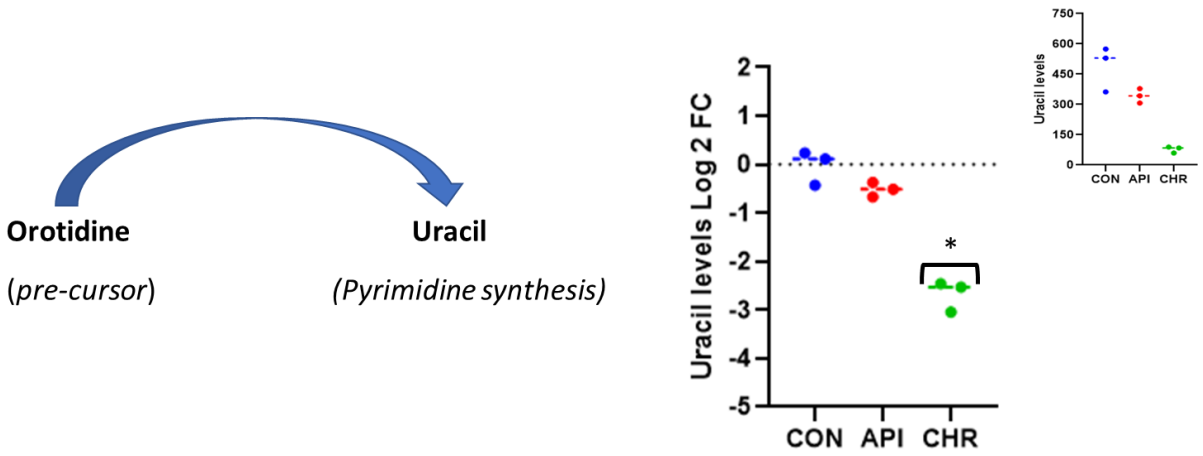
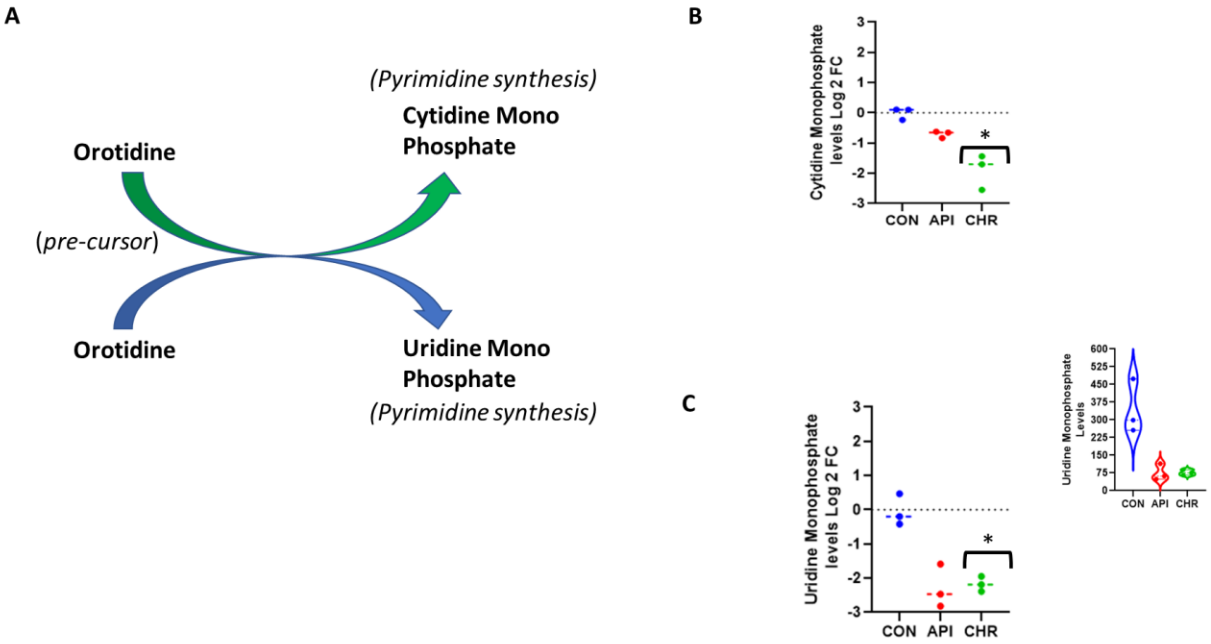


Supplementary figure – 3



Supplementary figure – 4

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