In vitro assay design using chicken liver extracts to study the CYP450-mediated metabolism of drugs & pharmaceuticals: a cheaper alternative for laboratories.

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Metabolism of xenobiotics such as drugs and pharmaceuticals mostly occurs in the liver where different metabolites are generated due to the biotransformation of xenobiotics by the cytochrome P450 (CYP450) family of enzymes. Metabolite evaluation is critical in the preclinical drug discovery in order to model the pharmacokinetics and pharmacodynamics (PK/PD). Most often the CYP450 assays are expensive and are not affordable by academic laboratories. In this study we designed an affordable *in vitro* assay using chicken liver extracts (ChiLEx) as a source of CYP450 and evaluated the metabolism of a most commonly used non-steroidal anti-inflammatory drug, paracetamol (acetaminophen) during the recent COVID-19 pandemic. ChiLEx was incubated with five different concentrations of paracetamol ranging between 0.01 gm/ml to 1.0 gm/ml. Our proton NMR spectra clearly demonstrated the metabolites of paracetamol. Further, the human known CYPs of paracetamol metabolism were used to find homologs in ChiLEx using Bioinformatics tools in order to validate the biotransformation of paracetamol by the ChiLEx. This assay is performed with a significantly lower cost compared to other standard methods.

Keywords: Liver extract, CYP450, Paracetamol, Acetaminophen, NMR, Proton spectrum.

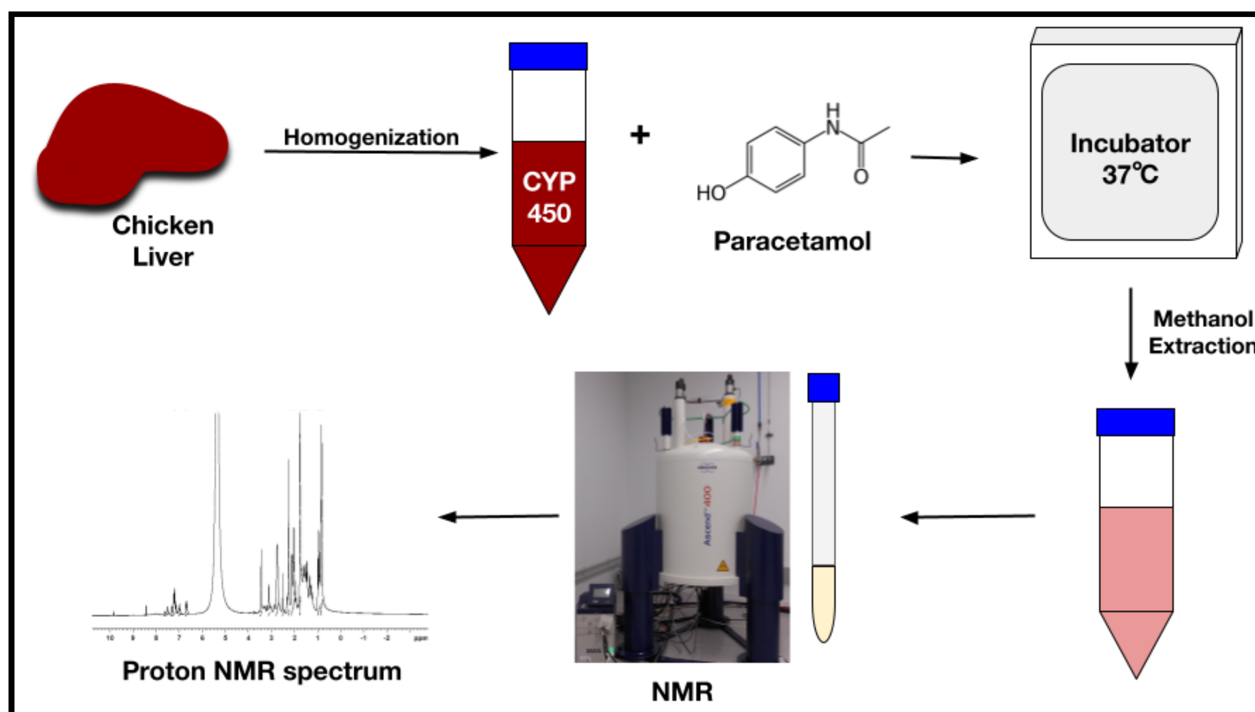

Figure 1. Overall process of analyzing paracetamol metabolism using chicken liver extract.

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Drug metabolism occurs after a particular drug is administered into the body. They get absorbed by intestinal epithelial cells, pass into the bloodstream through the hepatic portal system, get metabolized by the liver enzyme cytochrome P450 family, and show their effect on the target organ. These are later eliminated by the kidneys. Drugs are metabolized by oxidation, reduction, hydrolysis, hydration, conjugation, condensation, or isomerization. The enzymes involved in metabolism are generally present in most of the tissues, but are more concentrated in the liver as it is the primary site for drug metabolism. Drug metabolism is the biotransformation of drugs by enzymes within the body. These enzymes are divided into two groups, phase 1 and phase 2 enzymes; where Phase 1 enzymes are responsible for oxidation, reduction, and hydrolysis reactions (for example cytochrome P450, alcohol dehydrogenases), and Phase 2 enzymes catalyze conjugation reactions by the addition of functional groups to the intermediate metabolite (for example methyltransferases, glucuronosyltransferases, and glutathione S-transferases). The end-product of these metabolic processes will then be excreted through urine and bile.

CYTOCHROME (CYP) P450 ENZYMES: CYP is a membranous protein of the endoplasmic reticulum responsible for endogenous functions throughout the body. They alone account for up to 75% of the drug metabolism in the body. There are currently 19 CYP families classified in the invertebrates. The CYP protein is classified into superfamily (based on gene homology), gene family (CYP enzymes with at least 40% of amino identity) is denoted by a number- e.g., CYP3 and subfamily (CYP enzymes with at least 55% of amino identity) is denoted by an additional letter- e.g., CYP3A. Another number is used to indicate isoforms within the CYP subfamily- e.g., CYP3A1. The functions of CYPs include the production of steroid hormones, xenobiotic metabolism, bioactivation & deactivation, detoxification, fatty acid metabolism, catabolism of exogenous compounds, etc. CYPs play an important role in xenobiotic metabolism, which includes the conversion of non-natural or synthetic compounds (drugs and their components) into the metabolites that can be used by the body and the compounds that can be excreted easily by living organisms. Drug bioactivation and detoxification are essential and crucial as the accumulation of drug compounds can be harmful to the human body.

CYP MEDIATED DRUG METABOLISM: We picked paracetamol/acetaminophen (APAP) as a model substrate, a commonly used pain reliever, fever reducer, and well-known for its drug-induced hepatotoxicity at high doses. The toxicity is due to the highly reactive toxic intermediate, *N*-acetyl-*p*-benzoquinone imine (NAPQI), while the other metabolite, 3-hydroxy-acetaminophen (3-OH-APAP), is not toxic. APAP is mostly metabolized by CYP1A2, CYP2A6, CYP2E1, CYP3A4, CYP2C9, and CYP2D6. Among them, CYP2E1 is widely accepted as the principal source of NAPQI while CYP1A2 and CYP3A4 are also believed to contribute to NAPQI accumulation. On the other hand, CYP2A6 and CYP3A4 contribute to the conversion of APAP to 3-OH-APAP[1].

Stages of the catalytic cycle of CYP enzymes [2]: Binding of the substrate to the CYP enzyme, induces a conformational change and a spin transition of the heme iron. The initial reduction reaction of the heme group (Fe^{3+} to Fe^{2+}) when NAD(P)H transfers an electron to heme via the electron transfer chain which forms Fe^{2+} and then oxygen binds to the Fe^{2+} heme group, forming $Fe^{2+}O_2$. This becomes a more stable form, $Fe^{3+}O_2$. In the second reduction reaction, $Fe^{3+}O_2$ forms $Fe^{3+}O_2^{2-}$. The O_2^{2-} from $Fe^{3+}O_2^{2-}$ then reacts with two protons, breaking the bond between the two oxygen molecules and forming $(FeO)^{3+}$. The heme-bound oxygen atom is then transferred to the substrate. Finally, the product is released and enzymes are set free to start another cycle.

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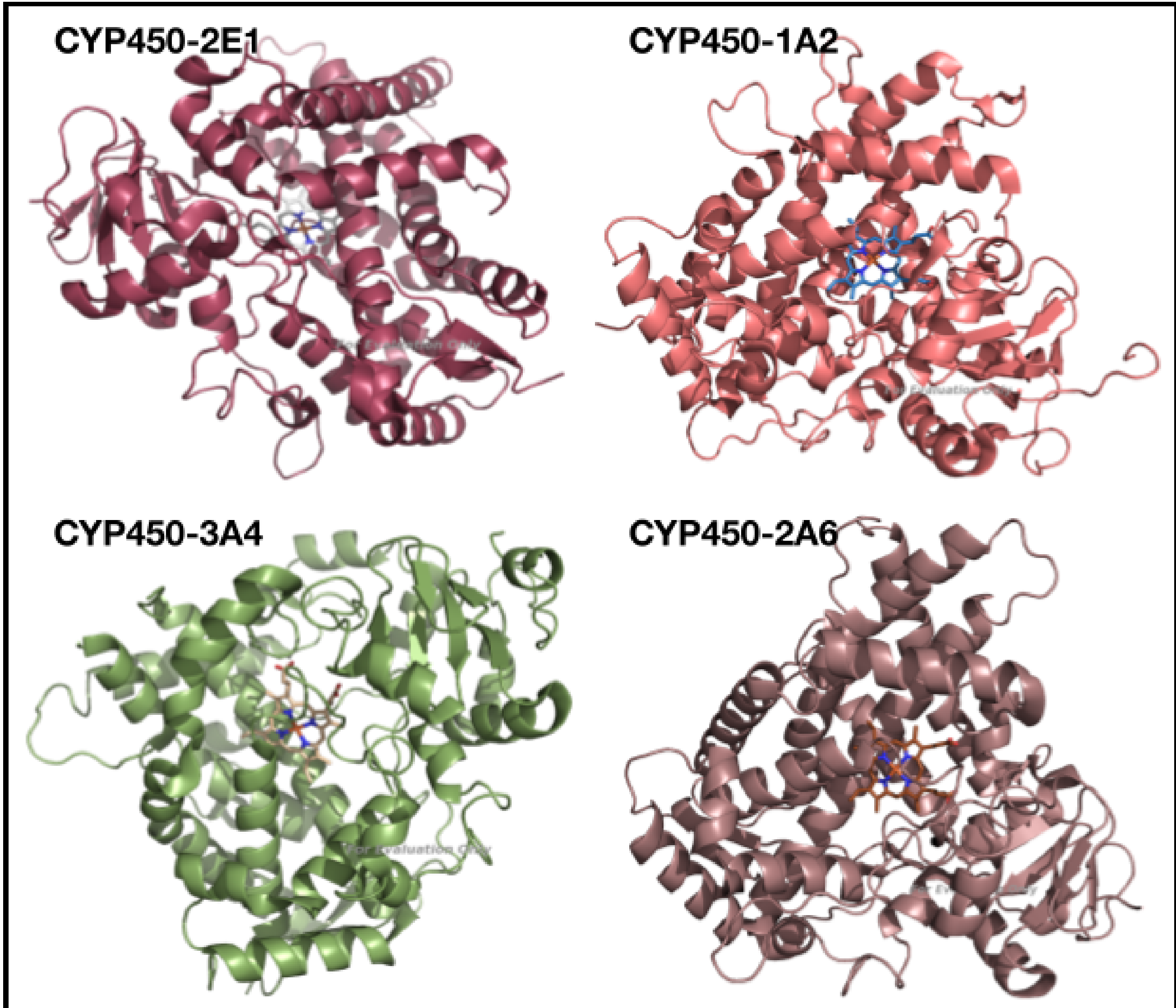

Figure 2. CYP450s involved in paracetamol metabolism.

CYP proteins differ in their expression levels and activity as drug administration can inhibit or induce the expression and activity of the drug metabolizing enzymes (DMEs). This in turn affects the rate of metabolism of each drug in the body affecting i.e., the duration of action for each drug. DDIs also effectively alter drug metabolism. The inhibition of CYP enzymes occurs through the binding of a non-substrate compound to its active site, reducing the possible activity. It is divided into three major categories:

- Reversible inhibition (competitive inhibition): Inhibitor will compete with the substrate for the active site of the CYP enzyme, blocking the normal catalytic activity.
- Quasi-irreversible inhibition: Inhibitor forms a complex with the heme moiety in the CYP enzyme making it inactive. This inhibition can also be reversed in vitro.
- Irreversible inhibition (suicide inhibition): Inhibitor will covalently bind to the heme site of the CYP rendering it inactive. The binding complex formed is irreversible and the protein loses its activity.

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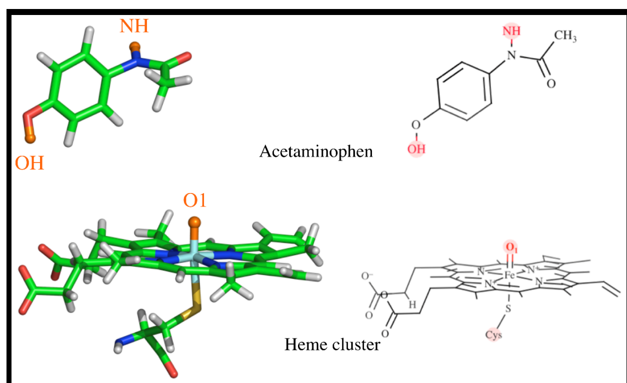

Figure 3. Paracetamol/acetaminophen & heme cluster in CYPs.

PARACETAMOL: Paracetamol/acetaminophen is the most popular analgesic/antipyretic drug molecule, also known as acetaminophen used for the activation of serotonergic pathways, inhibition of prostaglandin synthesis, activation of cannabinoid receptors; differential influence on transient receptor potential (TRP) channels, voltage-gated Kv7 potassium channels, T-type Cav3.2 calcium channels, and nitric oxide (NO) synthesis pathway. Thus, it is a multidirectional drug whose mechanism of action and effects have not been confirmed along with its safety concerns. Paracetamol can be ingested as a parent chemical but it may also be generated from phenacetin through a Phase I reaction involving CYP 1A2, another CYP isoenzyme different from 2E1 and with other substrate specificities[3]. The therapeutic use of paracetamol can cause severe liver toxicity if taken in large amounts. The recommended dose of acetaminophen for adults is 650 mg to 1000 mg every 4 to 6 hours, not to exceed 4 grams/day. In children, the dose is 60 mg/day. Toxicity develops at 7.5 g/day to 10 g/day or 140 mg/kg[4]. Acetaminophen toxicity is the second most common cause of liver transplantation worldwide and fifty percent of these are unintentional overdoses.

CYP-mediated drug metabolism of Paracetamol: Paracetamol has an analgesic effect in the CNS, mediated through the activation of its serotonergic pathways. The primary site of action may be the inhibition of prostaglandin (PG) synthesis or through the formation of an active metabolite that activates cannabinoid receptors. Prostaglandin H2 synthetase (PGHS) is the enzyme that metabolizes arachidonic acid into an unstable PGH2. There are two different forms of this enzyme, the constitutive PGHS-1, and the inducible PGHS-2. PGHS consists of two sites: a COX site and a POX site. The tyrosine-385 radical mediates the conversion of arachidonic acid to PGG2 at the COX site. The formation of a ferryl protoporphyrin IX radical cation from the reducing agent Fe3+ at the POX site is essential for the conversion of tyrosine-385 to its radical form. Paracetamol acts as a reducing co-substrate on the POX site and lessens the availability of the ferryl protoporphyrin IX radical cation. This action can be reduced in presence of hydroperoxide-generating lipoxygenase enzymes within the cell (called peroxide tone) or by saturating the POX site with a PGG2 substrate. This explains the lack of peripheral analgesic effect, platelet effect, and anti-inflammatory effect of paracetamol.

Acetaminophen shows adverse effects like protein denaturation, lipid peroxidation, and DNA damage when overdosed due to the generation of toxic metabolites when oxidized by reactive oxygen species. It is partially metabolized by hepatic microsomal CYP 2E1, CYP1A2, and CYP3A4. In the CYP-dependent paracetamol degradation, the toxic pathway gets activated if paracetamol in conjugation with sulfate and glucuronide (perform 90% of degradation) is exhausted, especially during a drug overdose. The toxic pathway leads to the formation of ROS and other toxic intermediates such as N-Acetyl-PBenzoquinone Imine (NAPQI), hydroquinone, p-aminophenol, p-nitrophenol, 1,4-benzoquinone. NAPQI is a toxic substance, reduced by glutathione to nontoxic mercaptate and other cysteine compounds, which are then excreted through the renal system. Drug overdose depletes the glutathione and once they reach less than 30% of the normal concentration, NAPQI levels increase.

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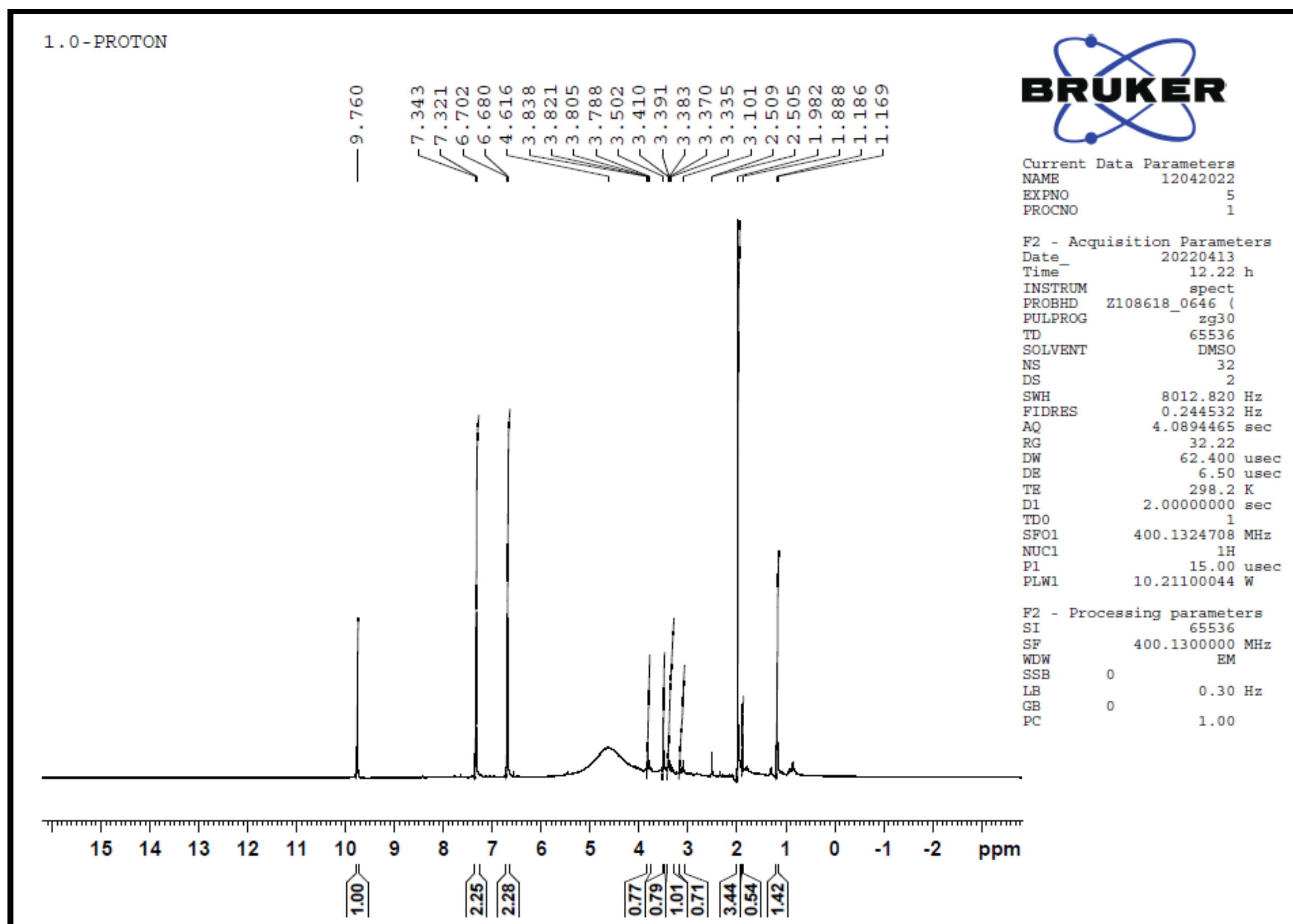

Figure 4. Proton NMR spectrum of paracetamol (1mg/12ml).

These NAPQI binds to hepatic macromolecules causing hepatic necrosis which is irreversible. They are detoxified by combining with hepatic glutathione and with the exhaustion of glutathione, these toxic intermediates bind to cell macromolecules and initiate mitochondrial injury, apoptosis, and liver cell necrosis. In this study we used chicken liver extracts (ChiLEx) as a cheap source of CYP450 enzymes to evaluate the metabolism of paracetamol/ acetaminophen at various drug concentrations. Post incubation of the drug with ChiLEx, we employed the proton NMR spectroscopy to evaluate the proton peaks.

Materials & Methods:

NCBI Search: The National Center for Biotechnology Information is a collection of sequences from different sources, including GenBank, RefSeq, SwissProt, PIR, and PDB which represent the data of a gene, genome, and transcript sequence that provide the foundation for research and discovery to determine the biological structure and function. Details of all the CYP450 enzymes in this study including their nucleotide FASTA sequences were obtained from the Gene database of the NCBI.

Multiple sequence alignment: The nucleotide sequences of CYPs in humans were aligned against each other using the CLUSTAL -OMEGA server to understand the length and conserved domains within these sequences. The search contains specific classes of cytochrome P450 in humans (*Homo sapiens*). The FASTA formats of CYP protein transcripts focused on in this search are the CYP1A2, CYP2A6, CYP2C9, CYP2D6, CYP2E1, and CYP3A4.

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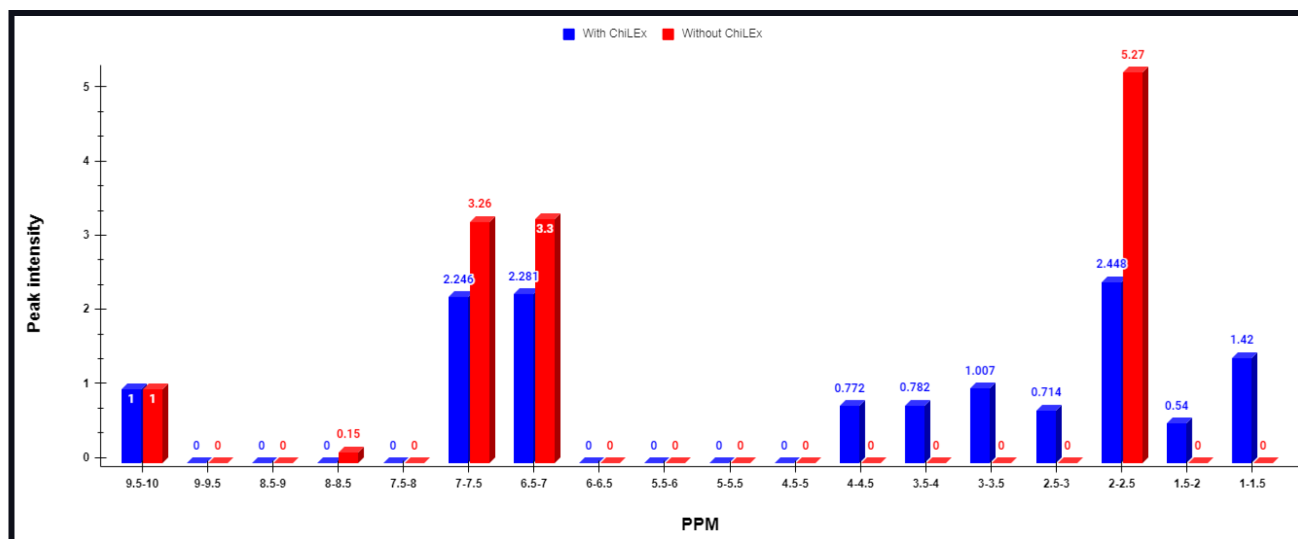

Figure 5. Proton NMR peaks with and without ChiLEx.

MSA was also performed for the CYPs commonly found in humans and chickens (CYP 1A2/2A6/3A4).

Preparation of ChiLEx: The overall protocol was modified from an earlier published protocol [6]. Briefly, the chicken liver is homogenized (mixing and blending of the sample) under sterile conditions into a slurry or paste using a mortar and pestle. Small amounts (7 ml was used in this study) of pre-chilled 1X TAE buffer was used in the homogenization of the liver. The homogenate obtained is then collected and distributed equally into 5 centrifuge tubes. The volume in each tube was made upto 6ml with pre-chilled 1X TAE buffer. The drug chosen for testing the liver metabolism is paracetamol. One paracetamol 650mg tablet was taken and homogenized into powder. The powder is then weighed and taken in different concentrations (0.01g, 0.10g, 0.25g, 0.5g, 1g) distributed into 5 tubes containing the diluted liver homogenate (ChiLEx), such that each tube has different drug concentrations: Control (1g of drug alone without ChiLEx); Test1 (0.01g of drug with 6ml of ChiLEx); Test2 (0.10g of drug with 6ml of ChiLEx); Test3 (0.25g of drug with 6ml of ChiLEx); Test4 (0.5g of drug with 6ml of ChiLEx) and Test5 (1g of drug with 6ml of ChiLEx). These sample tubes are incubated overnight at 37° C.

The tubes are taken out from the incubator after 20hrs of incubation. To each tube, equal amounts (6 ml) of 60% methanol was added and mixed thoroughly. These suspensions were boiled at 95°C for 5 min with shaking. The boiled suspensions were cooled to room temperature and then centrifuged at 13,000 rpm for 10 min to separate debris from the solvent extract. Supernatants were transferred into new tubes and were evaporated overnight to obtain dried samples and ready to be used for NMR spectroscopic analysis.

NMR Spectroscopy: Deuterated Dimethyl sulfox -ide (DMSO-d6) was added to each of the dried tubes. BRUKER Ascend 400 magnet was used for obtaining the proton NMR spectra. NMR spectra were acquired and processed using Top Spin software. Each proton spectrum is extended on a scale of 16 ppm to -4 ppm on the x-axis with the peak intensity being plotted on the y-axis (Figure 4). All the integrated proton peaks were analyzed using standard positional references for various types of protons.

Results and Discussion:

In vitro assay using ChiLEx as a cheaper alternative: The goal of this study is to develop

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and utilize broiler chicken liver microsomes to evaluate the metabolism and DDIs with drugs of interest, paracetamol/acetaminophen in this study. As shown in Figure 5, the proton NMR peaks of paracetamol/acetaminophen show significant difference not only in the number of peaks but also in the peak intensity when evaluated in the presence and absence of ChiLEx using the same concentration of the drug in both cases.

This also helps us to understand more deeply the differences in the CYP enzymes of humans and chicken during the drug evaluation and to build a model fulfilling the variations. This would improve the time and efforts for testing newly developed drugs easily without any further longing for the human liver samples. We hypothesize that liver microsomes obtained from healthy broiler chickens can be used as an effective *in vitro* tool for the measurement of drug metabolism based on drug depletion in microsomal incubations, and the identification of potential DDIs using drug combinations of interest. Taken together it is evident that the different concentrations of drugs such as paracetamol can be effectively metabolized by chicken liver extracts and hence this assay can be used in the future to evaluate other COVID-19 drugs such as favipiravir, remdesivir, molnupiravir, etc. However, this assay has to be further developed and optimized before using it for other drugs including combinations of drugs.

References

1. Yang Y, Wong SE, Lightstone FC (2014) Understanding a Substrate's Product Regioselectivity in a Family of Enzymes: A Case Study of Acetaminophen Binding in Cytochrome P450s. PLOS ONE 9(2): e87058.
2. Simmons, Hannah. (2019, February 26). What are Cytochrome P450 Enzymes? News-Medical. Retrieved on April 24, 2022 from https://www.news-medical.net/life-sciences/What-are-Cytochrome-P450-Enzymes.aspx.
3. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2584094/Bibi Z. (2008). Role of cytochrome P450 in drug interactions. *Nutrition & metabolism*, *5*, 27. https://doi.org/10.1186/1743-7075-5-27
4. Ye H, Nelson LJ, Gómez Del Moral M, Martínez-Naves E, Cubero FJ. Dissecting the molecular pathophysiology of drug-induced liver injury. World J Gastroenterol. 2018 Apr 07;24(13):1373-1385.
5. Paracetamol (Acetaminophen): mechanisms of action - ANDERSON - 2008 - Pediatric Anesthesia - Wiley Online Library https://doi.org/10.1111/j.1460-9592.2008.02764.x
6. Yedidi *et al.* P2' benzene carboxylic acid moiety is associated with decrease in cellular uptake: evaluation of novel nonpeptidic HIV-1 protease inhibitors containing P2 bis-tetrahydrofuran moiety. Antimicrob Agents Chemother. 2013 Oct; 57 (10): 4920-7. doi: 10.1128/AAC.00868-13.

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Conflict of interest: This research article is an ongoing project currently at TCABS-E, Rajahmundry, India. The authors invite collaborations without any conflict of interest.

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