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Assessment of CYP3A enzyme modulation by ethanolic skin extract of Duttaphrynus Melanostictus: An *In vitro* study

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Abstract
Cytochrome P450 3A4 (CYP3A4) is a predominant drug-metabolizing enzyme responsible for the biotransformation of a wide range of xenobiotics and therapeutic agents. The present study aimed to evaluate the inhibitory potential of Indian toad ethanolic skin extract (ETSE) on CYP3A4 activity using liver and intestinal microsomal systems. The incubation mixture containing microsomal suspension, erythromycin as substrate, and phosphate buffer was treated with varying concentrations of ETSE (5, 50, 300, and 2000 mg/mL). The reaction was initiated by NADPH and terminated using trichloroacetic acid. Formaldehyde formation was quantified spectrophotometrically at 412 nm using Nash reagent, and CYP3A4 activity was calculated from a formaldehyde standard calibration curve. The results demonstrated a concentration-dependent inhibition of CYP3A4 activity in both hepatic and intestinal microsomes. The highest concentration of ETSE (2000 mg/mL) produced marked inhibition comparable to the standard inhibitor ketoconazole. These findings indicate that Indian toad ethanolic skin extract possesses significant CYP3A4 inhibitory activity and may influence drug metabolism. Further studies are warranted to isolate active constituents and to evaluate the clinical and pharmacokinetic implications of this interaction.

Keywords: CYP3A4, Indian Toad, ethanolic skin extract, microsomes, drug metabolism, enzyme inhibition, herb–drug interaction, Ketoconazole

Introduction

Collection and isolation of ETSC

Toads (40–50 g) were collected from March to November within the Kakatiya University campus and its adjoining areas. The animals were thoroughly cleansed with tap water and selected according to the specified body weight range (40–50 g). Euthanasia was performed by pithing using a sterile pithing needle in accordance with standard amphibian handling procedures [1]. The skin was meticulously excised, carefully excluding the parotid gland region. The excised skins were immersed in mETSE and maintained at ambient temperature for 30 days in amber-colored glass bottles to prevent photodegradation of bioactive constituents [2]. Upon completion of the extraction period, the supernatant was decanted, centrifuged to eliminate residual particulate matter, and subsequently concentrated to dryness using a rotary evaporator under reduced pressure. The resulting residue was further air-dried at room temperature in sterile Petri dishes. The dried extract was preserved in a desiccator at ambient conditions until further experimental use. Duttaphrynus melanostictus skin extract (ETSE) was prepared from the Indian toad, *Duttaphrynus melanostictus* (formerly *Bufo melanostictus*) (common Indian toad) [3]. Previous investigations have demonstrated that ETSE possesses a broad spectrum of biological and pharmacological activities, notably including anticancer and anti-inflammatory properties [4, 6].

Sample preparation: ETSE is prepared into different concentrations by dissolving into normal saline solution and vortexed to form uniform solution before administration and stored at 40C.

In vitro CYP3A activity

The *in vitro* assessment of CYP3A activity was conducted in accordance with the method previously described by Nash *et al*. (1953) [7]. This assay is predicated upon the catalytic conversion of erythromycin by CYP3A into N-demethyl erythromycin and formaldehyde. The liberated formaldehyde subsequently reacts with Nash reagent to yield a yellow chromophore, the intensity of which is directly proportional to enzymatic activity [8].

The present investigation was undertaken to elucidate the *in vitro* modulatory effect of ETSE on CYP3A activity using a rat model. CYP3A constitutes a principal drug-metabolizing enzyme responsible for the biotransformation of a wide spectrum of therapeutic agents and xenobiotics [9]. Alterations in its activity can significantly influence substrate metabolism and consequently affect systemic bioavailability [10].

The findings of the study demonstrated that ETSE exerts a modulatory influence on CYP3A activity, producing a dose-dependent reduction in enzymatic function. These results indicate that ETSE significantly attenuates CYP3A activity with increasing concentrations, thereby suggesting its potential to interfere with CYP3A-mediated drug metabolism [11].

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1. Materials and methods

Erythromycin, employed as the specific substrate for CYP3A, was procured from Vasudha Pharma Chemicals Ltd., Hyderabad, India. Ketoconazole, used as the reference inhibitor, was obtained from Sigma–Aldrich (USA). Sucrose, Tris–HCl buffer, calcium chloride ($CaCl_2$), dipotassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), glycerol, histidine sucrose buffer (HSB), formaldehyde, and trichloroacetic acid were purchased from SD Fine Chemicals, Mumbai, India. NADPH was obtained from SRL Chemicals, Hyderabad, India.

All reagents and chemicals used in the study were of analytical grade and utilized without further purification.

2. Preparation of liver microsomes

Male Wistar rats were utilized for the experimental procedures. The animals were fasted for 24 hours prior to sacrifice. Livers were excised from both normal and atherogenic rats (n = 3 per group), promptly washed in ice-cold saline, and processed for microsomal preparation. The hepatic tissue was finely minced and homogenized in 5 mL of ice-cold 0.25 M sucrose solution containing 10 mM Tris–HCl buffer (pH 7.4). The homogenate was initially centrifuged at 600 × g for 5 minutes to remove cellular debris and nuclei, followed by centrifugation at 12,000 × g for 10 minutes to sediment the mitochondrial fraction. The resulting post-mitochondrial supernatant was carefully collected. To precipitate microsomes, calcium chloride ($CaCl_2$) was added to the supernatant to achieve a final concentration of 52 mM (0.2 mL per mL of supernatant). The mixture was allowed to stand for 15 minutes and subsequently centrifuged at 20,000 × g for 15 minutes. The resulting microsomal pellet was resuspended in a solution containing 150 mM KCl and 10 mM Tris–HCl, followed by centrifugation at 20,000 × g for 20 minutes to obtain a characteristic pink-colored microsomal pellet. Finally, the microsomal fraction was suspended in 0.5 mL of 0.1 M potassium phosphate buffer containing 20% glycerol and stored at −20°C until further experimental analysis. (Amar *et al*., 1974 and Tao *et al*., 2007).

3. Preparation of Intestinal microsomes

Intestinal microsomes were prepared with slight modifications to previously described procedures (Michaud *et al*., 2007; Sabatini *et al*., 2014). The freshly isolated intestine was sectioned into small segments and thoroughly rinsed with ice-cold phosphate-buffered saline to eliminate residual and unabsorbed luminal contents. The intestinal segments were then incised longitudinally to expose the mucosal surface in its entirety. The mucosal layer was gently and uniformly scraped from all segments, and the collected scrapings were pooled. The pooled mucosal scrapings were initially centrifuged at 25 × g for 5 minutes. The supernatant was discarded, and the resulting pellet was resuspended in 5.0 mL of ice-cold Histidine Sucrose Buffer (HSB) comprising histidine (5 mM, pH 7.0), sucrose (0.25 M), and Na-EDTA (0.5 mM; pH adjusted to 7.4). The suspension was homogenized and subsequently centrifuged at 15,000 × g for 10 minutes. The supernatant was carefully decanted into a clean centrifuge tube. The residual pellet was again resuspended in 5.0 mL of HSB and subjected to centrifugation at 15,000 × g for 10 minutes. Supernatants obtained from both centrifugation steps were pooled. To precipitate the microsomal fraction, calcium chloride ($CaCl_2$) was added to the combined supernatant to achieve a final concentration of 52 mM (0.2 mL per mL of supernatant). After allowing the mixture to stand for 15 minutes, it was centrifuged at 20,000 × g for 15 minutes. The resulting microsomal pellet was resuspended in 0.5 mL of 0.1 M potassium phosphate buffer containing 20% glycerol and stored at −20°C until further experimental use. The protein concentration of the microsomal fraction was quantified using the Biuret method, with bovine serum albumin (BSA) employed as the reference standard.

4. Erythromycin-N-demethylation assay (EMD Assay)

A reaction mixture containing microsomal suspension (0.1 mL, 25%), erythromycin (0.1 mL, 10 mM), and potassium phosphate buffer (0.6 mL, 100 mM, pH 7.4) was prepared and incubated at 37 °C in the presence of ETSE at concentrations of 5, 50, 300, and 2000 µg/mL. Ketoconazole (10 mM) was included as a positive control.

The enzymatic reaction was initiated by the addition of NADPH (0.1 mL, 10 mM) and allowed to proceed for 10 minutes. The reaction was terminated by the addition of ice-cold trichloroacetic acid (0.5 mL, 12.5% w/v), followed by centrifugation at 2000 × g for 10 minutes to precipitate and remove proteins.

An aliquot (1 mL) of the resulting supernatant was mixed with 1 mL of Nash reagent (comprising 2 M ammonium acetate, 0.05 M glacial acetic acid, and 0.02 M acetylacetone) and incubated in a water bath at 50 °C for 30 minutes. After cooling to room temperature, the absorbance was measured at 412 nm using a UV–visible spectrophotometer.

CYP3A4 activity was quantified using a standard calibration curve constructed with formaldehyde standards (1–100 µM), prepared by substituting the sample with known concentrations of formaldehyde and processed in parallel under identical conditions [12]. Enzyme activity was expressed as micromoles of formaldehyde formed per milligram of protein per hour [13]. Protein concentration was determined according to the method described by Lowry *et al*., enabling normalization of enzymatic activity to protein content [14].

Formula for calculating: CYP 3A Activity = Amount of CHO produced *(n/mol)* 1/25 mg of protein× 1/10 min

In vitro CYP 3A Activity - *Erythromycin*-N-demethylase (EMD) assay.

Table 1: Standard graph for formaldehyde (µM)

S.no	Concentration µM	Absorbance
1	10	0.032
2	20	0.141
3	40	0.285
4	80	0.592
5	100	0.785
6	120	0.846

Table 2: CYP3A activity in liver and intestine microsomes

S.no	CYP activity in liver microsomes (nM/mg/protein/min)					CYP activity in Intestine microsomes(nM/mg/protein/min)			
1	Group	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
2	Vehicle	562.2	101.7	415.22	8.19	484.1	20.22	512.71	11.33
3	Ketoconazole	138.5	2.988	122.76	14.11	211.6	4.95	121.3	6.88
4	ETSE 5mg/ml	424.5	2.986	362.81	5.46	465.2	40.1	382.8	23.29
5	ETSE 50mg/ml	367.8	3.164	315.1	2.20	344.2	34.8	364.5	13.53
6	ETSE 300mg/ml	266.8	19.16	221.41	40.61	268.2	11.5	222.2	13.12
7	ETSE 2000mg/ml	151.2	13.21	143.4	2.63	148.2	23.4	135.5	6.82

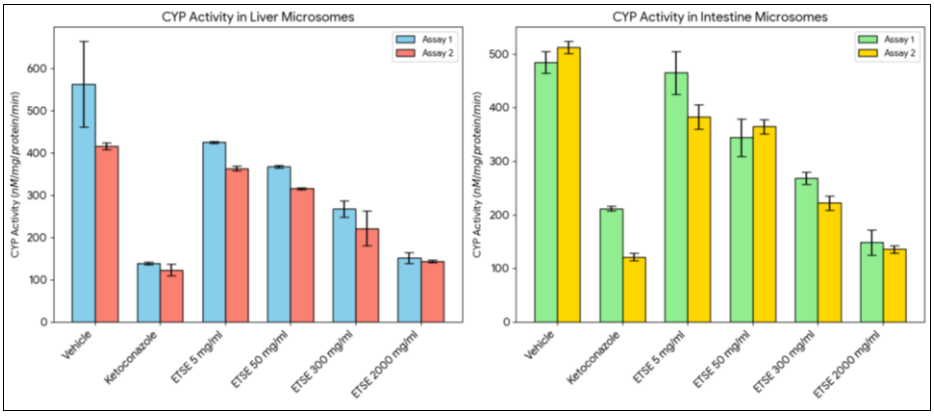

Fig 1: CYP Activity liver and intestine microsomes

Results and Discussion

The effect of ETSE on CYP activity in liver and intestinal microsomes is presented in Table X. CYP activity in the vehicle-treated group was 562.2 ± 101.7 nM/mg protein/min in liver microsomes and 484.1 ± 20.22 nM/mg protein/min in intestinal microsomes. Ketoconazole, used as a positive control, markedly reduced CYP activity to 138.5 ± 2.99 nM/mg protein/min in liver microsomes and 211.6 ± 4.95 nM/mg protein/min in intestinal microsomes, confirming significant inhibition of CYP activity. ETSE produced a concentration-dependent decrease in CYP activity in both liver and intestinal microsomes. At 5 mg/mL, CYP activity was reduced to 424.5 ± 2.99 nM/mg protein/min in liver microsomes and 465.2 ± 40.1 nM/mg protein/min in intestinal microsomes. Further reductions were observed at 50 mg/mL (367.8 ± 3.16 and 344.2 ± 34.8 nM/mg protein/min in liver and intestine, respectively). At 300 mg/mL, CYP activity declined substantially to 266.8 ± 19.16 nM/mg protein/min in liver microsomes and 268.2 ± 11.5 nM/mg protein/min in intestinal microsomes. The highest concentration tested (2000 mg/mL) produced pronounced inhibition, with CYP activity decreasing to 151.2 ± 13.21 nM/mg protein/min in liver microsomes and 148.2 ± 23.4 nM/mg protein/min in intestinal microsomes, approaching the inhibitory effect observed with ketoconazole. Overall, ETSE demonstrated a dose-dependent inhibitory effect on CYP activity in both hepatic and intestinal microsomes, with more pronounced inhibition observed at higher concentrations.

Conclusion

The findings of the present investigation demonstrate that the ethanolic skin extract of Indian toad (ETSE) exhibits significant inhibitory activity against CYP3A4 in both liver and intestinal microsomes. The extract produced a clear concentration-dependent reduction in CYP activity, with higher concentrations showing marked inhibition comparable to the standard inhibitor, ketoconazole. These results indicate that ETSE possesses potent modulatory effects on CYP3A4-mediated metabolism. Given the pivotal role of CYP3A4 in the biotransformation of a wide range of xenobiotics and therapeutic agents, the observed inhibitory potential of the Indian toad ethanolic skin extract suggests possible herb–drug interaction risks, as well as potential applications in modulating drug metabolism.

Future Scope

Future studies should focus on:

1. Isolation and characterization of active constituents responsible for CYP3A4 inhibition using advanced chromatographic and spectroscopic techniques.
2. Mechanistic studies to elucidate the mode of inhibition (competitive, non-competitive, or mechanism-based inhibition).
3. *In vivo* pharmacokinetic investigations to assess the clinical relevance of CYP3A4 inhibition and potential drug–extract interactions.
4. Toxicological evaluation to establish safety profiles and therapeutic windows.
5. Molecular docking and in silico studies to predict binding interactions with the CYP3A4 enzyme.

Such investigations would provide deeper insight into the pharmacological significance, safety considerations, and possible therapeutic applications of Indian toad ethanolic skin extract.

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