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ORIGINAL ARTICLE

In vitro UDP-Glucuronosyltransferase and Cytochrome P450 Enzymes Activities of *Clinacanthus nutans* Leaf Juice and Aqueous Extract

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ABSTRACT

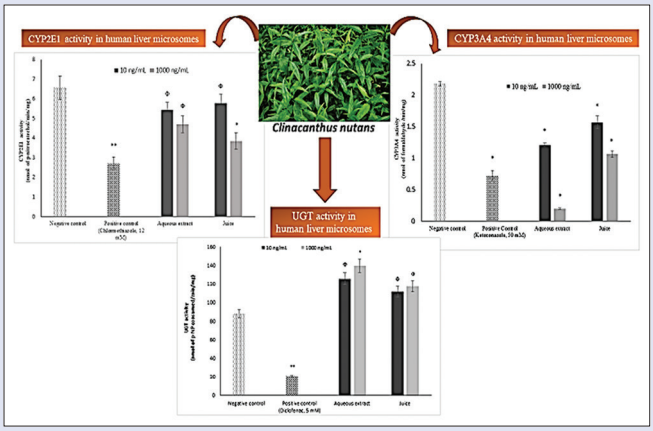
Aim: The objective of the present study was to evaluate the *in vitro* effect of aqueous extract of *Clinacanthus nutans* leaves and the juice on the activity of UDP-glucuronosyltransferase (UGT), cytochrome P (CYP) 3A4, and CYP2E1 in human liver microsomes (HLMs). **Materials and Methods:** The herb-drug interactions of the leaf extracts and juice were determined by a specific enzyme activity of CYP isoforms with specific probe substrate using spectrophotometry. CYP3A4 activity was measured for aminopyrine-specific metabolite (formaldehyde) at 415 nm. CYP2E1 activity was determined using *p*-nitrophenol-specific metabolite (*p*-nitrocatechol) at 535 nm. UGT activity was quantified through the consumption of *p*-nitrophenol by UGT at 405 nm. **Results:** Results obtained showed that the juice and aqueous extract of *C. nutans* leaves exhibited significant inhibition ($P < 0.05$) in CYP3A4 and CYP2E1 activity in HLMs. The aqueous extract of *C. nutans* showed statistically significant ($P < 0.05$) activation on UGT activity at the concentration of 1000 ng/mL as compared to the negative control. **Conclusion:** There is a possibility that herb-drug interaction could occur with *C. nutans* through inhibitory effects on CYP3A4 and CYP2E1. The leaf preparation also activated UGT catalyzed metabolism which may result in a reduction of the potency of the drug metabolized by UGT pathway.

Key words: *Clinacanthus nutans* leaf, cytochrome P2E1, cytochrome P3A4, extracts, human liver microsomes, UDP-glucuronosyltransferase

SUMMARY

- The study revealed that *Clinacanthus nutans* leaf preparations may interact with cytochrome P (CYP) 3A4 and CYP2E1 enzymes and it should be used with caution with drugs that metabolized by these enzymes to avoid potential adverse effects. This *in vitro* study using human liver microsomes provides important drug interaction screening and research information on safety during the interaction between the herb and drug.

Abbreviations used: ATR: Attenuated total reflectance; CYP: Cytochrome P; HLMs: Human liver microsomes; IR: Infrared; TFC: Total flavonoids content; UGT: UDP-glucuronosyltransferase; NaOH: Sodium hydroxide; NADPH: Nicotinamide adenine dinucleotide phosphate; PBS: Phosphate-buffered saline; $ZnSO_4$: Zinc sulfate; $Ba(OH)_2$: Barium hydroxide.



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INTRODUCTION

Herb-drug interaction can be defined as any alteration either in the pharmacokinetic or pharmacodynamic effect of the drug caused by concurrent treatment with herb and drug. Interaction of drugs with Phase I and Phase II liver enzymes may reduce the efficacy of the drug and increase its toxicity.[1,2] Phase I liver enzymes, Cytochrome P (CYP) 3A4 is responsible for the most marketed drug metabolism pathway, and CYP 2E1 is accountable for activation of a numbered of carcinogens.[3,4] The CYP2E1 enzyme promotes its detoxification physiological role and to prevent the production of reactive oxygen intermediates. Phase II enzyme UDP-glucuronosyltransferase (UGT) conjugates metabolites from Phase I oxidation to assist in its elimination.

Enzyme-inducing and inhibitory effects by herbal medicinal products are often undetected and result in a potential inadequate therapy or observed side effects; hence, there are concerns with the use of herbal medicines, including potential herb-drug interaction. Bioactive constituents from plants have a specific physiological action on human enzymes, including the interaction with human liver enzymes.[5,6]

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Green tea catechin showed a significant increase in buspirone, a CYP phenotypic indices that suggest a reduction of CYP3A4 activity in humans.[5] Cichoric acid, a phenolic compound of *Echinacea purpurea* plant, has been reported for its herb-drug interaction with CYP3A4.[2] Green tea catechins and polyphenols in milk thistle extract have been reported to inhibit Phase I enzymes, including CYP3A4 and CYP2E1.[7] Vinca alkaloids, vinblastine isolated from *Catharanthus roseus* had been shown to induce CYP3A4.[8]

In our previous study, methanol leaves extract of *Clinacanthus nutans* was found to exhibit inhibitory effects on CYP3A4 and CYP2E1, which indicates probable anti-carcinogenesis effects in human liver microsomes (HLMs).[9] Many herbal medicinal products are aqueous-based extracts or the juice from the leaves, fruits, and seeds. The present study describes *in vitro* effect of aqueous leaf extract and fresh leaf juice of *C. nutans* on UGT and Cytochrome P450 3A4 and 2E1 enzymes activity in HLMs.

MATERIALS AND METHODS

Chemicals and reagents

Chemicals used were aminopyrine, *p*-nitrophenol phosphate, methanol, ketoconazole, *p*-nitrocatechol, formaldehyde, aluminum chloride, and sodium nitrite (Acros Organics, USA), Catechin hydrate (Sigma-Aldrich, Japan), sodium nitrite, sodium hydroxide (NaOH) (Friedemann Schmidt Chemical, UK).

Plant material

Approximately 6.5 kg fresh leaves of *C. nutans* were collected from a private commercialized herbal park known as Yik Poh Ling Herbal Farm, Persatuan Pengkaji Herbal Tradisional, Pantai, Seremban, Negeri Sembilan. The voucher specimen was prepared and identified at the Institute of Bioscience, University Putra Malaysia: *C. nutans* (SK 2208/13). Five hundred gram of fresh leaves of this plant were kept for juice processing. The remaining leaves were washed thoroughly under tap water and air-dried at 37°C for 7 days. The weight of the dried leaves was measured and grounded to the powder using a blender (Waring, USA). The leaves powder was kept in an air-tight bottle for further use in the subsequent process for extraction.

Preparation of aqueous extract from the dry leaf

Aqueous extracts of *C. nutans* was prepared by weighing 75 g of dry pulverized leaves and macerated with 300 ml distilled water in a conical flask. The mixture was placed in a hot water bath set at 100°C for 30 min. Then, the mixture was left to cool to room temperature and filtered after 24 h. The extraction was repeated under the same condition until colorless extract was obtained. The extracts were filtered and then freeze-dried. The yield of extraction was calculated, and the extract was stored in desiccators until further used for the study.

Preparation of fresh leaf juice

Fresh leaves juice of *C. nutans* was prepared by direct grinding (without adding any solvent) of 100 g fresh plant leaves of each. The leaf juice was collected in an airtight clean bottle and freeze-dried. The yield of extraction was calculated. The extract was stored in an air-tight bottle for further use within 24 h.

Total flavonoid content

The total flavonoid content (TFC) in *C. nutans* preparation was quantified using aluminum chloride colorimetric assay by a spectrophotometer according to the method of Pekal and Pyrzyniska.[10] Absorbance was measured at 510 nm by spectrophotometer, and distilled water was used as a blank. Catechin (20–100 mg/L) was used as the standard, and the TFC of each preparation was expressed as milligram catechin equivalent per gram of the dry weight of the sample. The experiments were performed in triplicates.

In vitro cytochrome P3A4 activity

The human hepatic drug metabolizing activity, CYP3A4, was determined by measuring the formaldehyde released from *N*-demethylation of its substrate aminopyrine based on the method as described by Nicholas *et al.*[11] The experimental test group was subjected to three different concentrations ranging from 10 to 1000 ng/mL, with the number of samples equal to 3 (n = 3), and 0 ng/mL as the control for all herbal preparations. The positive control used for CYP3A4 assay was ketoconazole (50 nM). Each group containing HLMs (1 mg/mL) was pretreated with 0.01 mL TritonX-100 (0.375% v/v) for 3 min at 37°C to offset the membrane latency before the experiment. Incubation medium consisted of HLMs (1 mg/mL), aminopyrine (14 mM), nicotinamide adenine dinucleotide phosphate (NADPH), phosphate buffer (0.1 M; pH 7.4), and *C. nutans* extract at different concentrations (10–1000 ng/mL). After 5 min of incubation, the chemical reaction was terminated by adding 0.01 mL of 25% (w/v) zinc sulfate followed by saturated barium hydroxide solution. The mixture was allowed to stand for 5 min before centrifugation at 1000 × *g* for 5 min. The supernatant obtained was added into the Nash reagent and preceded the incubation at 60°C for 30 min in a shaking water bath. Approximately 1 mL of distilled water was added to the aliquot from a tube and transferred to a cuvette to measure the absorbance at 405 nm using a spectrophotometer. The concentration of formaldehyde formed from CYP3A4-mediated *N*-demethylation of aminopyrine was calculated from the standard curve of formaldehyde plotted. The CYP3A4 activity was expressed in nmol formaldehyde formed/min/mg protein.

In vitro cytochrome P2E1 activity

The CYP2E1 activity was determined by measuring *p*-nitrocatechol formed from the *p*-nitrophenol hydroxylation.[12,13] The positive control used for CYP2E1 assay was chlormethiazole (12 mM). Incubation medium consisted of HLMs (1 mg/mL), *p*-nitrophenol (5 mM), NADPH, 0.1 M phosphate-buffered saline pH 7.4, and *C. nutans* extract at different concentrations (10 and 1000 ng/mL). The reaction began with the addition of cofactor, NADPH, to each group for 30 min for enzyme reaction at 37°C. The reaction was terminated after 30 min by adding 20% (v/v) trichloroacetic acid and mixed thoroughly. The mixture was then kept in ice for 5 min. The reaction medium was then centrifuged at 10,000 × *g* for 5 min. The residual supernatant obtained was added to a clean Eppendorf tube containing 2 M NaOH and vortexed. Approximately 1 mL of distilled water was added to the aliquot from the tube and transferred to a cuvette to measure the absorbance immediately at 515 nm using a spectrophotometer. The absorbance value obtained was compared to the standard curve of *p*-nitrocatechol to identify the concentration of *p*-nitrocatechol formed from the reaction.

In vitro UDP-glucuronosyltransferase activity

The human Phase II hepatic drug-metabolizing activity, UGT was determined by measuring the *p*-nitrophenol disappearance to ether glucuronide that formed from *p*-nitrophenol glucuronidation.[14] Method and incubation condition used for UGT assay were prepared in accordance with the literature report with slight modification.[15] Since the metabolite of glucuronidation takes inversion of alpha and beta form, the assay of UGT consecutively used the concept for its indicator for activity by measuring the consumption of the substrate,

p-nitrophenol in NaOH color reagent. The reaction was initiated by treating incubation of the medium containing the pretreated HLM in 0.02 mL of 30 mM uridine diphosphate-glucuronic acid for 15 min at 37°C. The total volume of the reaction mixture was 0.2 mL. The reaction was terminated by adding 0.08 ml of 20% w/v trichloroacetic acid to precipitate the protein. The reaction mixture was centrifuged at 2000 × *g* for 10 min. Then, 0.2 mL supernatant was mixed with 0.8 ml of 5 M NaOH to develop the light yellowish color of the mixture. The absorbance of *p*-nitrophenol consumed was measured at 405 nm. The amount of *p*-nitrophenol consumed through *O*-glucuronidation process in HLM was calculated from the standard curve of *p*-nitrophenol. The calibration solution concentrations of 0, 12.5 μM, 62.5 μM, 125 μM, 312.5 μM, 625 μM, 1250 μM, and 2500 μM were prepared from a 5 mM stock solution. Then, *p*-nitrophenol was transferred into test tubes containing the complete incubation mixture but with heat-inactivated enzymes. The blank was 1 ml of distilled water. The absorbance was measured at 405 nm. Each measurement was made in triplicate. Diclofenac (5 mM) was used as a positive control. The UGT activity was then expressed in nmol *p*-nitrophenol consumed/min/mg protein.

The absorbance of the amount of product formed, or probe consumed in case of UGT activity

(U) was calculated using the equation below:

U = (Experimental group − Blank group) − (Control group − Blank group).

The positive control, diclofenac (5 mM), was included to assess the test validity in UGT assay. All test groups were pretreated with 0.008 mL Triton-X100 (0.75% v/v) instead of 0.01 mL Triton X-100 (0.375% v/v) as per other tests for 3 min to offset the membrane latency prior to the experiment.

Attenuated total reflectance Fourier transform infrared fingerprints

Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectra of the aqueous extract and the juice were obtained with Nicolet™ iS5 FTIR spectrometer controlled by OMNIC software (Thermo Fisher Scientific, Waltham, Massachusetts, USA) for spectra collection and TQ Analyst software for data processing (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The instrument is equipped with iD5 ATR accessory featuring a top plate diamond crystal with a fixed angle of incidence of 42°. The qualitative FTIR spectroscopy analysis was conducted in the mid-IR range of 4000–400/cm at a resolution of 4/cm with 14 scans. The background spectrum was recorded before obtaining the spectra of the samples.

Data analysis

Data were presented as mean ± standard error of the mean. The data for the present study were analyzed using GraphPad Prism 5 (GraphPad Software, San Diego, California, USA) using analysis of variance following Dunnett's test to examine the significant differences of each treatment group (test and positive control group) compared to the negative control group. With a confidence level of 95% ($P < 0.05$), the difference would be considered as statistically significant when the treatment group was compared to the control group.

RESULTS AND DISCUSSION

Extraction yield and total flavonoid contents

The percentage yields and TFCs of the fresh leaves juice and dried leaves aqueous extracts of *C. nutans* are shown in Table 1. The percentage yield of the aqueous extract was greater than juice. The high yield of *C. nutans* in water extracts was probably due to the high solubility of major components of *C. nutans* in the polar solvent. The yields of fresh juices of 11 herbs were reported to be lower than those of ethanol and water extracts.[16] Thus, mechanical forces in the form of pressing and squeezing fresh leaves in preparing leaves preparation may be inferior to the chemical solvent extraction method. Although mechanical forces allowed leaves to breakdown and release the liquid soluble secondary metabolites, chemical extraction methods have higher efficiency by using aqueous and methanol solvent to improve the yield of extraction.[17,18]

The flavonoid contents of the two preparations were significantly different [Table 1]. TFC was higher in the leaf juice than the cold aqueous extract. Despite the higher yield of the aqueous extract, its TFC was lower than that of the juice. Total flavonoids content of *C. nutans* herbal tea prepared by using microwave oven dried method was reported to be lower than that of freeze-dried method suggesting that drying process can contribute to the loss in flavonoids.[19] Flavonoids have the potential to modulate CYP450 activities which can lead to the production of environmental carcinogens. Thus, the presence of flavonoids in *C. nutans* preparations may contribute to CYP3A4 and CYP2E1 and UGT enzymes activity. Therefore, the *in vitro* UGT and CYP 450 enzymes activities in HLMs were investigated.

In vitro cytochrome P3A4 activity

The change in CYP3A4 hepatic drug metabolizing enzyme activity of aqueous leaf extract and leaf juice of *C. nutans* was evaluated. Preliminary experiment to optimize assay condition for CYP3A4 showed linearity in HLM activity. The assay for CYP3A4 conducted showed positive results in positive control. Figure 1 shows the inhibitory effect of *C. nutans* on CYP3A4 through aminopyrine *N*-demethylation in HLMs at the concentrations tested. Fresh leaf juice and aqueous leaves extract of *C. nutans* at 10 ng/mL and 1000 ng/mL showed statistically significant ($P < 0.05$) inhibitory effect on CYP3A4 in HLMs as compared to the control. Differences in the

Table 1: The percentage yield and total flavonoid content of *Clinacanthus nutans* leaves preparations

	Cold aqueous extract	Fresh leaves juice
Weight of leaves (g)	75.00	305.98
Percentage yield of extraction (% w/w)	6.87	0.55
Total flavonoid content (mg catechin Eq/g dry weight)	245.48	283.33

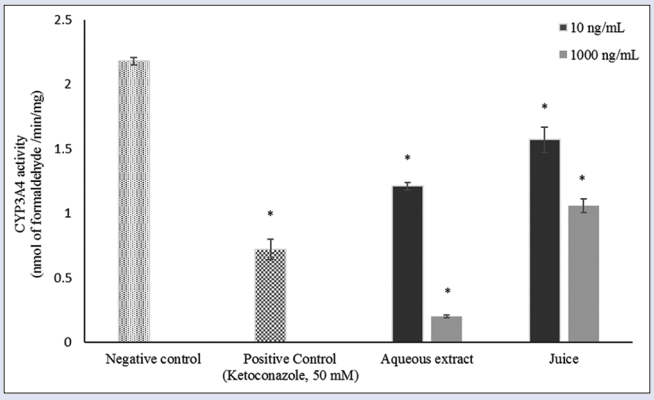

Figure 1: *In vitro* effect of leaf juice and aqueous extract of *Clinacanthus nutans* on the activity of cytochrome P3A4 in human liver microsomes. $N = 3$; data values were presented as mean ± standard error of the mean; analyzed using Dunnett's test; *$P < 0.05$, a significant difference compared to the negative control

flavonoid content, the number of the hydroxyl group and types of interaction with CYP3A4 could contribute to higher inhibition effect of the cold aqueous extract on CYP3A4 as compared to the juice.

The inhibitory effect could be ascribed to its flavonoid constituents.[18,20,21] Inhibition of CYP3A4 in human had been well documented for flavonoids.[11,22] Despite the very large and flexible active site for CYP3A4, substrate binding was mainly based on hydrophobicity with some steric interactions.[20] Flavones and flavonols inhibit enzyme activity by directly binding to CYP3A4 isoforms that involved in xenobiotics metabolisms.[18] Flavones which bound to CYP3A4 undergo hydroxylation or demethylation to inhibit the enzyme activity. Inhibition of CYP3A4 will lead to xenobiotics that metabolized by the same enzyme to slow down in its metabolism. Thus, concomitant use of a drug metabolized through CYP3A4 and *C. nutans* preparation will cause the xenobiotics to remain in the body system for a longer period. This may potentially cause toxicity in those drugs with major side effects at a low dose. From data on flavonoid-CYP interactions, flavonoids that possess hydroxyl groups inhibit CYP activity, whereas those lacking hydroxyl groups can stimulate enzyme activity.[20,21]

In vitro cytochrome P2E1 activity

The CYP2E1 hepatic drug metabolizing enzyme (carcinogens activating enzyme) activity of the aqueous extract, and the juice was investigated by measuring the formation of *p*-nitrocatechol (product) from hydroxylation of *p*-nitrophenol (substrate) through *p*-nitrophenol hydroxylation of HLMs. The preliminary experiment to optimize assay condition for CYP2E1 showed linearity in the human liver microsomal activity. The assays for CYP2E1 conducted showed positive results for the positive control. The results of CYP2E1 activity is shown in Figure 2. Only the leaf juice at 1000 ng/mL showed a statistically significant ($P < 0.01$) inhibitory effect on CYP2E1 in HLMs as compared to the control group. The aqueous extract concentration at 1000 ng/mL showed a decrease in activity; however, the reduction in activity was not statistically significant ($P > 0.05$) when compared to the control value. Similarly, at the concentration of 10 ng/mL juice and aqueous extract demonstrated a reduction in CYP2E1 activity, which was not significant ($P > 0.05$) compared to control value. Only the juice at high concentration inhibited significant effect on CYP2E1 activity. The inhibition of CYP2E1 activity may have an inhibitory effect on carcinogenesis initiation.[23,24]

In vitro UDP-glucuronosyltransferase activity

The UGT activity of aqueous extract and juice of *C. nutans* leaves were measured from the consumption of *p*-nitrophenol (substrate) through *O*-glucuronidation process in HLMs which represented by *p*-nitrocatechol consumed/min/mg. Preliminary experiment to optimize assay condition for UGT showed linearity in HLM activity. The assay showed positive results in positive control. *C. nutans* preparations showed significant activation activity on UGT (Phase II hepatic drug metabolizing enzyme) in HLM [Figure 3]. The aqueous extract of *C. nutans* showed significant ($P < 0.05$) activation on UGT activity at the concentration of 1000 ng/mL as compared to the negative control. At the concentrations of 10 ng/mL, the juice and the aqueous extract showed activation of UGT activity, but the activation was not significant ($P > 0.05$) as compared to the control value. The activation of UGT activity indicates that simultaneous use of a drug metabolized through UGT and *C. nutans* preparations may result in the faster detoxification process and reduction in its potency. These extracts were found to contain flavonoids which have been reported to be associated with UGT induction in human hepatic cell lines and glucuronidation in UGT1A1 through a nonaryl hydrocarbon receptor-mediated mechanism.[18,20,25]

Attenuated total reflectance fourier transform infrared fingerprints

The ATR-FTIR fingerprints are useful for the identification of functional groups of compounds present in the samples based on the characteristic IR vibrations frequencies of the functional groups. ATR-FTIR spectra of the aqueous extract and the juice are shown in Figure 4. The ATR-FTIR fingerprints of the two samples were similar.

The spectra showed an intense hydroxyl (-OH) band at 3200–3600/cm, C-H stretch at a frequency range of 2900–3000/cm, Carbonyl (C = O) stretch at a frequency of 1605–1630/cm, and C-O stretch at a frequency of 1080–1050/cm. The frequency range of 3200–3600/cm was the stretching vibration for hydroxyl group for alcohol, including phenol. Besides that, the frequency of 1628/cm was assigned to the carbonyl group for carbonyl compounds. The presence of hydroxyl

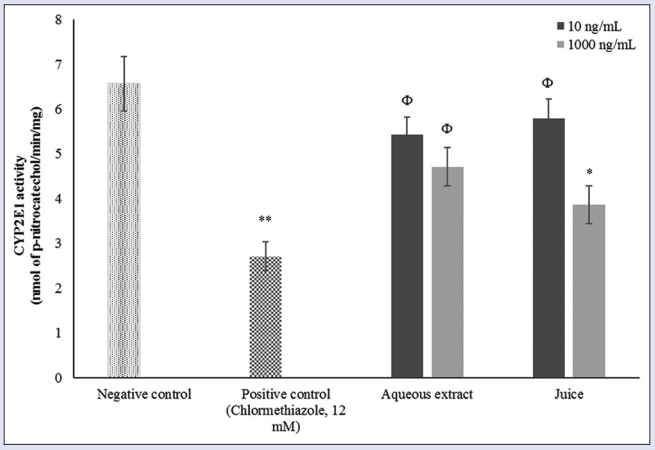

Figure 2: *In vitro* effect of leaf juice and aqueous extract of *Clinacanthus nutans* on the activity of cytochrome P2E1 in human liver microsomes. $N = 3$; data values were presented as mean ± standard error of the mean; analyzed using Dunnett's test; *$P < 0.05$, **$P < 0.01$ significant difference compared to the control and Φ no significant difference compared to the negative control

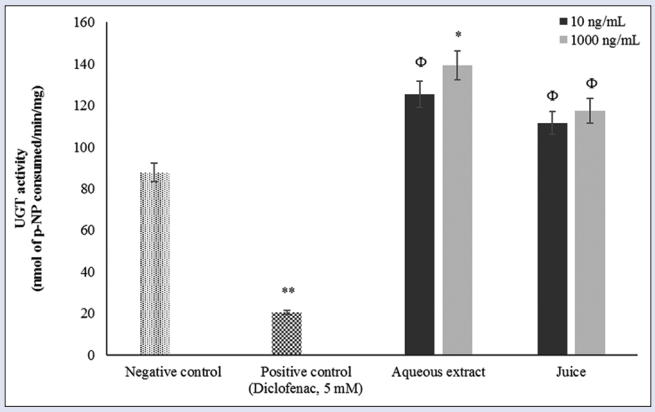

Figure 3: *In vitro* effect of leaf juice and aqueous extract of *Clinacanthus nutans* on the activity of UDP-glucuronosyltransferase in human liver microsomes. $N = 3$; data values were presented as mean ± standard error mean; analysed using Dunnett's test; *$P < 0.05$, **$P < 0.01$ significant difference compared to the negative control and Φ no significant difference compared to the negative control

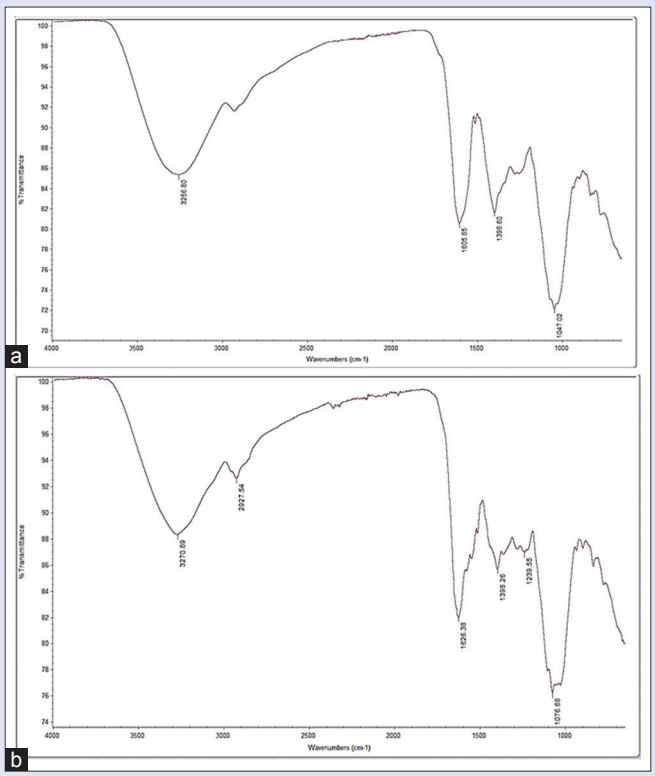

Figure 4: Fourier transform infrared spectra *Clinacanthus nutans* leaf. (a) aqueous extract; (b) juice

band and carbonyl stretch suggested compounds in the extracts have a hydroxyl functional group and carbonyl functional group. Phenolic and flavonoids compounds are compounds containing hydroxyl and carbonyl group as functional groups of chemical constituents in the extract. C-glycosyl flavones in the extract have a hydroxyl functional group.

CONCLUSION

C. nutans leaf preparations may interact with CYP3A4 and CYP2E1 enzymes, and it should be used with caution with drugs that metabolized by these enzymes to avoid potential adverse effects. The leaf preparation also activated UGT catalyzed metabolism which may result in a reduction of the potency of the drug metabolized by UGT pathway. Despite the findings of this study, the herbs could be safe for consumption since metabolism occurs through various enzymes than a single enzyme pathway. This *in vitro* study using HLM provides important drug interaction screening and research information by providing a fundamental understanding of safety during the interaction between the herb and drug. This *in vitro* study excludes drug transporter effect; nevertheless, the information can be beneficial in preclinical discovery stages in drug development. Data regarding the CYP450 inhibitory activities of *C. nutans* have shown the additional information needed to be considered in designing animal studies to predict the effect that might be anticipated in humans. *In vivo* effect of leaf extract and juice of *C. nutans* leaves on drug-metabolizing enzymes activity for quantitative measurement in quantitative prediction of human biotransformation are in progress.

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Conflicts of interest

There are no conflicts of interest.

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