IN VITRO XANTHINE OXIDASE INHIBITORY AND *IN VIVO* HYPOURICEMIC ACTIVITY OF DUAL HERBAL COMBINATION COMPRISING *CARICA PAPAYA* L. AND *PIPER LOLOT* C. DC LEAVES

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Hyperuricemia has emerged as a major public health issue in recent years, with existing treatments often associated with negative side effects for patients. This research examined the anti-hyperuricemic properties of a dual herbal extract made from Carica papaya L. and Piper lolot C.DC leaves, utilizing a xanthine oxidase inhibitory assay and a hyperuricemic mouse model induced by potassium oxonate (500 mg/kg body weight). The results indicated that a 5-day treatment with the hexane herbal extracts (100 and 300 mg/kg) significantly lowered serum and urine uric acid levels in hyperuricemic mice and inhibited xanthine oxidase activity in vitro (IC50 of 27.39 ± 0.32 µg/mL), which could be the underlying mechanism for the observed urate-lowering effects, as the extract did not promote increased uric acid excretion in urine. Our findings imply that the combined extract of Carica papaya L. and Piper lolot C. DC leaves may help alleviate gout by inhibiting uric acid production.

Keywords: *Carica papaya*, *Piper lolot*, uric acid, xanthine oxidase, potassium oxonate, mice.

I. INTRODUCTION

Xanthine oxidase (XO) is essential for the breakdown of purines in humans, leading to the production of uric acid. The enzymatic activity of xanthine oxidase facilitates the conversion of hypoxanthine to xanthine, which is subsequently transformed into uric acid. Ultimately, uric acid is excreted through urine. An overproduction of uric acid can lead to conditions such as hyperuricemia and gout.[1] Hyperuricemia is a significant risk factor for conditions such as gout, hypertension, and diabetes.[2] The underlying mechanism of the disease involves the crystallization and accumulation of uric acid in the joints and surrounding tissues. Since the overproduction of uric acid is the primary cause of hyperuricemia, targeting xanthine oxidase is essential for effectively treating this condition.[2,3]

Currently, numerous medications are available for the clinical management of elevated uric acid levels; however, these often come with a range of side effects. For instance, allopurinol can lead to headaches, allergic reactions, skin rashes, increased aminotransferase levels, nephritis, and other negative effects, making it unsuitable for patients with renal impairment. There is a pressing need to create effective and low-toxicity treatments for hyperuricemia.[2,3]

The use of herbal medicine has significantly increased recently, with many natural products being incorporated into the market to address

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public health issues. More than 80% of the global population relies on natural remedies to treat various ailments. However, many of these natural products remain untested, and there is inadequate information regarding their mechanisms of action and safety profiles.[4]

This study evaluated a dual herbal formulation consisting of *Carica papaya* (CP) and *Piper lolot* (PL) for its xanthine oxidase inhibitory activity. A subsequent experimental trial was carried out to assess its efficacy in mice with hyperuricemia.

Piper lolot C. DC is a sprawling herb that is part of the Piperaceae family. This herb has a long history of use in traditional medicine among Southeast Asian communities and is commonly referred to as 'Lolot' in Vietnam. Modern pharmacological research has demonstrated that crude extracts of *P. lolot* exhibit a diverse array of biological activities, including antibacterial, antifungal, anti-osteoporosis, antidepressant, neuroprotective, anti-inflammatory, anticancer, hypoglycemic, insecticidal, and antihypertensive properties.[5] A recent study indicated that the dried extract of lolot leaves effectively reduced uric acid levels in experimental animals without causing any toxic effects.[6]

Carica papaya L., commonly referred to as papaya, is a member of the Caricaceae family. This perennial horticultural shrub is native to the Mesoamerican region, including Central America and southern Mexico, and is primarily grown in tropical and subtropical areas. While papaya is well-known for its sweet, ripe fruit, various parts of the plant-such as seeds, leaves, roots, flowers, bark, and latex-have been traditionally utilized globally for medicinal purposes. Notably, the leaves have gained recognition for their numerous health-promoting properties.[7] Research conducted by Azmi SMN et al. (2012) utilized reversed-phase flash column chromatography (RPFCC) and high-performance thin layer chromatography (HPTLC) to isolate a xanthine oxidase inhibitor from papaya leaves, highlighting their potential benefits for individuals suffering from gout.[8]

The findings suggest that CP and PL leaves could serve as promising candidates for drug development to treat hyperuricemia. However, to our knowledge, the hypouricemic effects of a combined formulation of papaya and lolot leaf extracts in experimental animal models of hyperuricemia have not been thoroughly explored. Consequently, this study aims to investigate the potential hypouricemic effects of this dual herbal extract both *in vitro* and *in vivo*.

II. MATERIALS AND METHODS

1. Chemicals

N-hexane and ethanol were obtained from Fisher Scientific (UK). Potassium oxonate (PO), xanthine oxidase, and xanthine were sourced from Sigma (USA). Allopurinol was acquired from Stellapharm J.V. Co., Ltd. (Vietnam). The assay kits for measuring creatinine and uric acid were provided by ERBA Lachema S.R.O, Czech Republic.

2. Papaya and lolot leaf extract preparations

Excellent-quality fresh CP and PL leaves were harvested from papaya and lolot trees cultivated in Khoai Chau district of Hung Yen province. The leaves were botanically identified and authenticated at the Institute of Ecology and Biological Resources at the Vietnam Academy of Science and Technology. A voucher specimen (YD-ĐL2023) has been archived at the Department of Ethnobotany within the same institute in Hanoi, Vietnam.

The leaves were thoroughly washed under running tap water and shade-dried for five days before being ground into a fine powder

using an electric mixer. For extraction, 3.0kg of the powdered plant material, consisting of an equal mixture of papaya leaves and lolot leaves, was treated with 70% ethanol at 65°C. The crude herbal powder underwent three extraction cycles, each lasting three hours, with solvent-to-herbal material ratios of 10:1, 8:1, and 8:1 (w/w). The resulting solutions were filtered, combined, and then distilled under reduced pressure, producing a crude ethanol extract (CEE) weighing 709.4g. A portion of the crude residue, weighing 700.1g, was further fractionated with n-hexane using one liter of solvent for each of the three applications. After this process, the solvent was evaporated, resulting in 217.6g of hexane extract (HXE). The extracts were diluted in distilled water for oral administration before use.

3. Animals

Fifty *Swiss* mice, approximately 11 weeks old and weighing between 20 and 25 grams, were supplied by the National Institute of Hygiene and Epidemiology located in Hanoi, Vietnam. These mice were maintained under standard laboratory conditions and provided with a consistent diet and unlimited access to water. They were acclimatized for 7 days before the initiation of the experiments. The study was conducted at the Department of Pharmacology, VNU University of Medicine and Pharmacy.

4. Xanthine oxidase inhibitory activity assay

The inhibitory activity of xanthine oxidase (XO) was assessed following the methodology outlined by Abu-Gharbieh E et al. (2018). Initially, the extracts were dissolved in dimethyl sulfoxide (DMSO) and then diluted with phosphate buffer at pH 7.5. The concentration required to inhibit 50% of the XO enzyme activity (IC50) for each extract was determined using a range of concentrations (5, 10, 25, 50, and 100 µg/mL). A mixture was prepared consisting of 50µL of the test solution, 35 µL of phosphate buffer, and 30µL of freshly prepared XO solution (0.1 U/mL), which was pre-incubated for 15 minutes at 25°C. Subsequently, 60µL of xanthine solution (750µM) was introduced to initiate the reaction. The final mixture was incubated for an additional 30 minutes at 25°C. The reaction was halted by introducing 25µL of a 1N HCl solution before measuring the absorbance by a Biotek ELISA Plate Reader. Allopurinol was utilized as a positive control, tested at various concentrations (0.08, 0.4, 2, and 10 µg/mL). The negative control (blank) was prepared similarly, with HCl added before the substrate. Each analysis was performed in triplicate, and the percentage of XO inhibitory effect was calculated using the appropriate formula:

Percentage of XO inhibition = 100 - [(OD_{sample} - $OD_{control}$) × 100]

Where $OD_{control}$ = $OD_{negative\ control}$ - OD_{blank}; OD_{sample} = OD_{sample} - OD_{blank}

The half-maximal inhibitory concentration (IC50) was determined using TableCurve2Dv4 software.

5. Mice model of hyperuricemia

PO was freshly suspended in 0.5% sodium carboxymethylcellulose (CMC-Na). The hyperuricemia mouse model was established by administering 500 mg/kg of PO intraperitoneally (i.p.) one hour before the final drug interventions for all groups, except for the NC group, which received an equivalent volume of 0.5% CMC-Na (i.p.).[10]

Drug administration: A total of 50 mice were randomly assigned to five separate groups, with each group containing 10 mice. The first group served as the control and did not receive any treatment or potassium oxonate injection. The second group was labeled as the hyperuricemia group, in which the mice were administered potassium oxonate only. The third group acted

as the positive control, receiving an oral dose of allopurinol at 20 mg/kg. The fourth and fifth groups were treated with the test extract at 100 mg/kg and 300 mg/kg, respectively. All treatments were given orally once a day for five days.

Biochemical Assay: After the last drug dose, urine samples from each mouse were gathered over five hours. The collected fluid was then centrifuged to determine the concentrations of uric acid and creatinine. After the urine collection was completed, blood samples were drawn from the carotid arteries. The blood was permitted to clot for about one hour at room temperature before being centrifuged at 3000rpm for ten minutes to separate the serum. The serum creatinine and uric acid levels were subsequently assessed using assay kits.

The fractional excretion of uric acid (FEUA) represents the ratio of urate filtered by the glomeruli to the amount excreted in the urine, expressed as a percentage. The assessment of FEUA was conducted using the equation outlined below:[11]

FEUA = ([serum creatinine×urine urate]/[urine creatinine×serum urate])×100

Statistical analysis

The data was collected using Microsoft Excel 2010, followed by statistical analysis performed with SPSS 26.0. Results are expressed as mean ± standard deviation (SD). A Student's t-test was employed to determine significant differences between the two groups, with a p-value of less than 0.05 indicating statistical significance.

III. RESULTS

In vitro xanthine oxidase inhibitory activity

Table 1. Xanthine oxidase inhibition of CEE and HXE

Concentration (µg/mL)	%Xanthine oxidase inhibition		
	Crude ethanol extract	Hexane extract	Allopurinol[a]
100	48.96 ± 0.15	88.56 ± 0.81	98.29 ± 1.97
50	27.21 ± 0.49	68.37 ± 1.04	88.87 ± 0.24
25	21.21 ± 1.01	45.69 ± 1.01	74.63 ± 0.63
10	19.15 ± 0.41	25.52 ± 1.29	53.92 ± 0.18
5	12.61 ± 0.36	12.45 ± 1.09	43.51 ± 0.42

[a]Allopurinol was tested at four distinct concentration ranges (10, 2, 0.4, and 0.08 µg/mL)

Both extracts, CEE and HXE, demonstrated over 50% inhibition of xanthine oxidase; however, their inhibitory effects were lower than that of the positive control, allopurinol, which showed an inhibition rate of 98.29 ± 1.97% (IC_{50} = 7.16 ± 0.07 µg/mL). At a concentration of 100 µg/mL, HXE achieved an inhibition of 88.56%, while CEE resulted in a 48.96% reduction in enzyme activity (refer to Table 1). The IC_{50} values for HXE and CEE were recorded at 27.39 ± 0.32 and greater than 100 µg/mL, respectively (see Chart 1). The n-hexane extract was identified as the more potent of the two, exhibiting the highest percentage of xanthine oxidase inhibition and the lowest IC_{50} value. Consequently, this extract was selected for further *in vivo* studies to evaluate its hypouricemic effects.

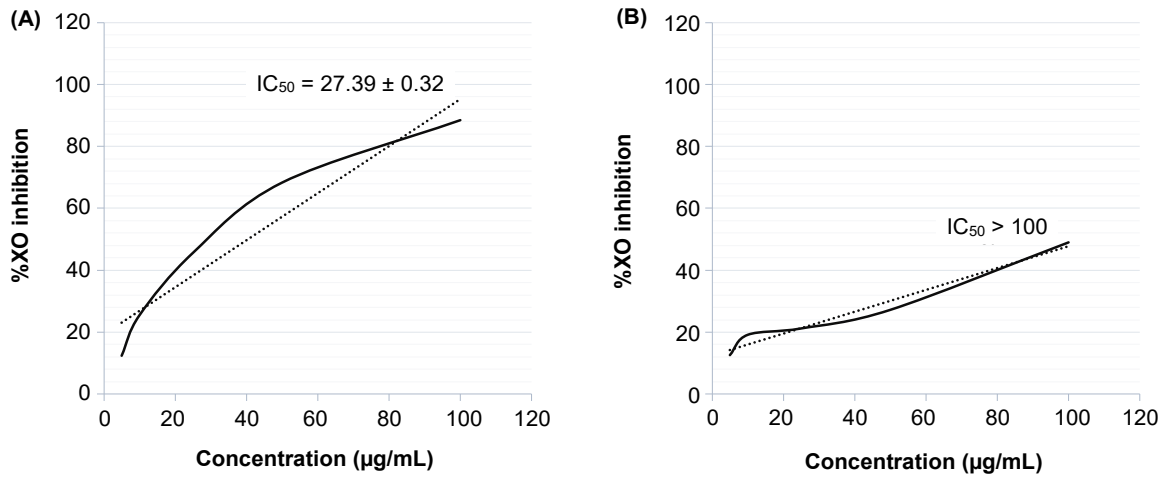
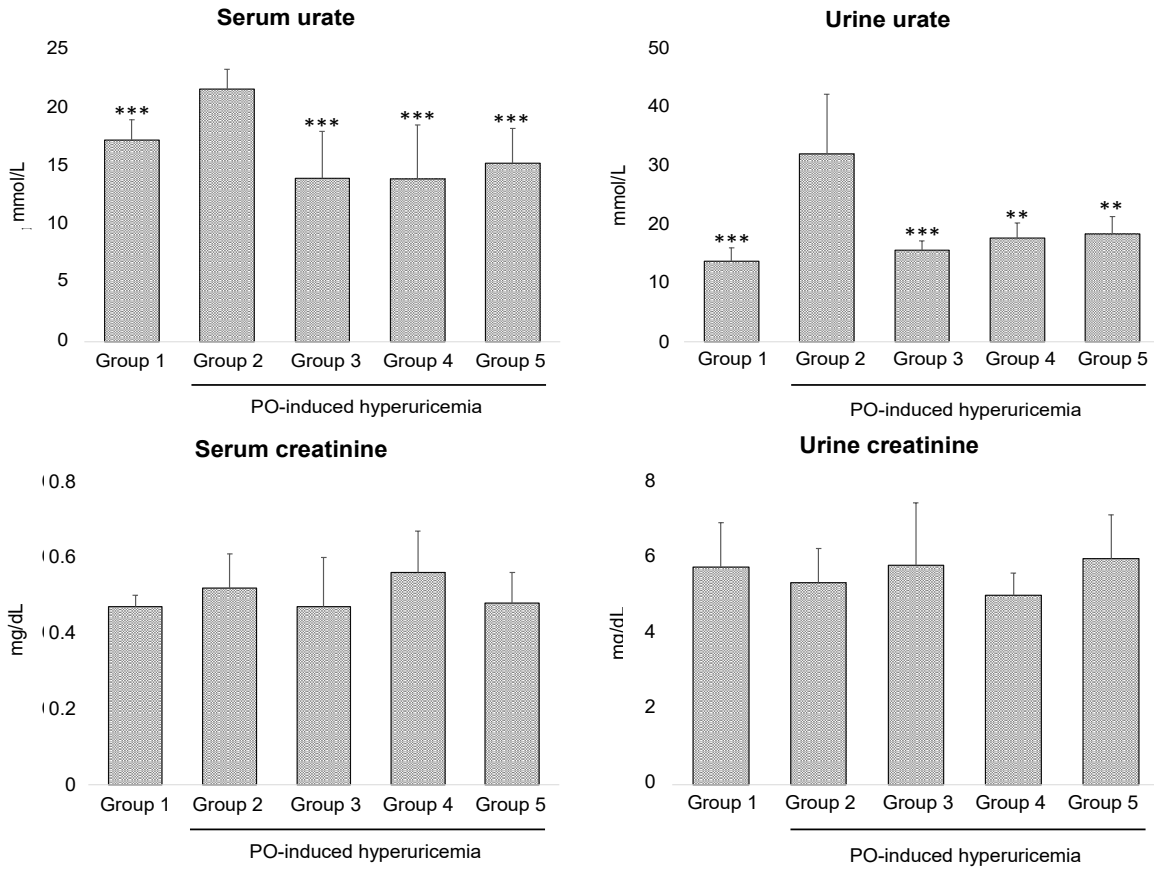


Chart 1. Representatives of %xanthine oxidase (XO) inhibition-concentration (µg/mL) curves and IC_{50} values of (A) hexane extract and (B) crude ethanol extract established through the *in vitro* xanthine oxidase inhibitory assay

In-vivo hypouricemic activity

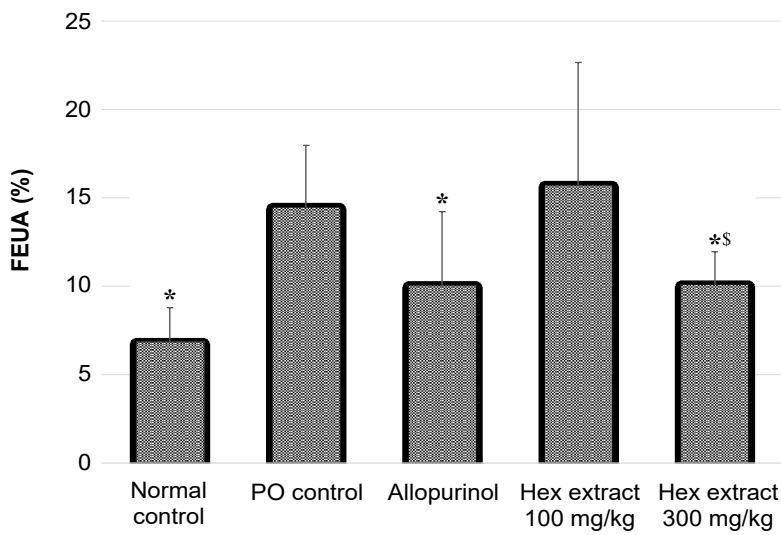
The plant extracts were administered orally at 100 and 300 mg/kg for 5 days to mice with hyperuricemia induced by PO. We assessed serum and urine uric acid levels using the uric acid assay detailed in the previous section. As illustrated in Chart 2, the serum uric acid concentration in the PO-induced hyperuricemic mice (Group 2) was significantly elevated compared to the healthy control group (1) ($p < 0.001$). In contrast, the allopurinol group (3), serving as the positive control, exhibited significantly reduced serum uric acid levels ($p < 0.001$). The groups treated with HEX (4 - 5) showed a decrease in serum uric acid levels at both low (100 mg/kg) and high (300 mg/kg) doses when compared to the hyperuricemic control. Notably, the uric acid-lowering effects of both extract dosages were comparable to those of allopurinol, with no significant differences ($p > 0.05$) observed between the effects of allopurinol and the plant extract treatments.

The creatinine concentrations in both serum and urine exhibited minimal variations across all animal groups (Chart 2). Notably, the fractional excretion of uric acid (FEUA) was significantly elevated in the hyperuricemia group compared to the control group ($p < 0.05$). Furthermore, treatment with HEX at the administered doses did not improve uric acid excretion; in fact, FEUA values were significantly reduced in the group receiving 300 mg/kg of HEX (Chart 3).



***p < 0.01; ***p < 0.001 as a significant difference compared with hyperuricemia group (Group 2)*

Chart 2. Effects of HEX on urate and creatinine levels in PO-induced hyperuricemic mice



**p < 0.05; $p < 0.05 as a significant difference compared with PO control and Hex extract 100 mg/kg, respectively*

Chart 3. Effects of HEX on the fractional excretion of uric acid

IV. DISCUSSION

While allopurinol, febuxostat, and benztropine are commonly prescribed for hyperuricemia, their adverse effects often deter patients from committing to long-term treatment, leading to low adherence rates.[2,3] There is a growing interest in utilizing natural products for managing hyperuricemia. In this study, we showed that hexane extracts from the leaves of *C. papaya* and *P. lolot* notably decreased xanthine oxidase activity *in vitro* and lowered serum uric acid levels in mice induced with hyperuricemia.

Xanthine oxidase inhibitors (XOIs) are crucial in managing hyperuricemia and gout by lowering circulating uric acid levels and reducing oxidative stress in the vascular system linked to high uric acid concentrations. The primary action of XOIs is to block the production of uric acid from purines within the body. Beyond pharmaceutical options, various herbs and their extracts have been historically utilized to address conditions stemming from increased xanthine oxidase activity. These natural treatments have demonstrated the ability to influence xanthine oxidase activity and may provide alternative strategies for controlling hyperuricemia and its associated disorders.[12] Our research did not demonstrate xanthine oxidase inhibitory activity in the crude ethanol extracts from papaya and lolot leaves. In contrast, the hexane extracts from both types of leaves exhibited this activity, with IC_{50} values of 27.39 ± 0.32 µg/mL. In a separate investigation, the essential oil extracted from the leaves of *P. lolot*, sourced from Thua Thien Hue province in Vietnam, demonstrated notable inhibitory effects on xanthine oxidase with an IC50 value of 28.4 ± 1.7 µg/mL, comparable to the inhibition observed in our research. Based on molecular docking analysis, the β-bisabolene component within this oil shows promise as a potential candidate for drug development for treating gout.[13] Additionally, *C. papaya* leaves were found to contain various secondary metabolites, predominantly flavonoids, alkaloids, saponins, xanthine alkaloids, terpenoids, and anthranol glycosides, which may contribute to their pharmacological effects, particularly in inhibiting xanthine oxidase activity.[8] At a concentration of 100 µg/ml, the EEA1 fraction (EtOH – EtAc fraction) derived from the distilled water extract of papaya leaves exhibited an inhibitory activity of 95.70 ± 2.57% against xanthine oxidase, surpassing the activity of allopurinol, which was measured at 93.69 ± 0.2%.[8] The inhibitory effect of this papaya leaf fraction was greater than the hexane fraction identified in our study (88.56 ± 0.81% at 100 µg/ml). This result underscores the significance of choosing an appropriate extraction solvent to optimize the active compounds in papaya leaf extract for future research endeavors.

As *in vitro* results, we further assessed hypouricemia activity of the dual herbal hexane extract in laboratory mice. Hyperuricemia in animals is induced by inhibiting the enzyme uricase, which is responsible for converting uric acid into allantoin through intraperitoneal injection of PO. Various researchers have utilized this model to investigate the anti-hyperuricemic effects of medicinal plants.[10,11] In the current study, serum uric acid levels were markedly increased following PO administration, confirming the successful establishment of the hyperuricemic model. In contrast, the group treated with allopurinol exhibited significantly reduced serum uric acid levels ($p < 0.001$). The hexane extract alleviated serum uric acid levels at both low (100 mg/kg) and high (300 mg/kg) dosages, demonstrating reductions of 35.6% and 29.2%, respectively, in comparison to the

hyperuricemic control group. *In vivo* studies have also demonstrated hypouricemic effects from individual extracts of lolot or papaya leaves.[6,14] Additionally, the administration of HEX at both high and low doses did not significantly enhance the renal handling capacity of fractional excretion of uric acid (FEUA). Consequently, it can be considered that HEX mitigated hyperuricemia, and this effect was not associated with increased uric acid excretion, indicating that it did not prevent uric acid deposition.

V. CONCLUSION

The hexane fraction of *Carica papaya* L. extract and *Piper lolot* C.DC extract demonstrated anti-hyperuricemic activities in experimental animal models and had the xanthine oxidase inhibitory activities in vitro. The hexane fraction of *Carica papaya* L. and *Piper lolot* C.DC extract demonstrated significant XO inhibitory activity *in vitro* with IC_{50} of 27.39 ± 0.32 µg/mL. Both doses of the Hexane fraction (100 mg/kg and 300 mg/kg) markedly reduced serum and urine uric acid levels in hyperuricemic mice. The uric acid-lowering effect of the fraction is likely associated with the mechanism of XO inhibition rather than being linked to the process of enhancing uric acid excretion in the urine.

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