Xanthine oxidase inhibitory activity from potential Malaysian medicinal plant as remedies for gout

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Abstract: Malaysia has a rich diversity of medicinal plants and some of them inhibit xanthine oxidase (XO), which can be introduced as new natural sources of gout medication and a substitute for synthetic xanthine oxidase inhibitors (XOI). The degree of XO inhibitory activity was determined by measuring the absorbance spectrophotometrically at 295 nm, which is associated with uric acid formation. Our preliminary screening study had employed the use of distilled water, 70% methanol and absolute ethanol to extract XOI from twenty parts of five plant species, namely, *Averrhoa carambola, Carica papaya, Dimocarpus longan malesianus, Manilkara zapota* and *Salacca zalacca*. These plants were selected based on their frequent medicinal usages by local folks. The results have shown that an aqueous extract of *Carica papaya* mature leaves has promising activity to inhibit XO up to 75.68 ± 0.1%. Statistical experimental design were employed to optimize the selected sample (dried *Carica papaya* leaves: distilled water) on extraction of XOI and the maximum XOI percentage of 86.93 ± 1.9% was obtained, which exhibited only 6.76% less than the activity exhibited by allopurinol (93.69 ± 0.2%), a commercial XOI. The comparison was made between allopurinol and optimized extract on the basis of IC_{50} concentrations. Allopurinol showed IC_{50} value of 3.74 µg/ml that is considerably lower as compared to the optimized sample (4.33 µg/ml).

Keywords: *Carica papaya*, xanthine oxidase inhibitor

Introduction

Enzymatic degradation of hypoxanthine and xanthine leads to the production of uric acid (González *et al*., 1995; Ramallo *et al*., 2006). Elevated concentrations of uric acid in the blood stream of human body (Tausche *et al., 2004)* leads to formation of gout, characterized by hyperuricemia and recurrent attacks of arthritis (Virsaladze *et al., 2007*). Xanthine oxidase (XO) is an enzyme responsible for catalyzing the oxidation of hypoxanthine to xanthine and of xanthine to formation of uric acid (Owen and Johns, 1999; Ramallo *et al*., 2006). XO is distributed most abundantly in the liver and intestine (Battelli *et al*., 1972), situated at the end of a catabolic sequence of the purine nucleotide metabolism in humans and few other uricotelic species (Unno *et al*., 2004). It generates superoxide (O_2^-) during oxidation of substrates (Battelli *et al*., 1972), subsequently plays an important role in various forms of inflammatory diseases (Rohman *et al*., 2010), several types of tissue and vascular injuries (Berry and Hare, 2004), and chronic heart failure (Pacher *et al*., 2006).

The treatment of gout entails the use of therapeutic agents such as xanthine oxidase inhibitors (XOI) (Kong *et al*., 2001; Unno *et al*., 2004). XOI acts by blocking the biosynthesis of uric acid from purine in the body (Unno *et al*., 2004) and it is believed that either by increasing the excretion of uric acid or reducing the uric acid production helps to reduce the risk of gout (Umamaheswari *et al*., 2007). Allopurinol is one of the many known synthetic XOIs, that is widely used in the therapeutic and clinical management of gout, conditions associated with hyperuricemia and related inflammatory diseases (Fields *et al*., 1996; Pacher *et al*., 2006). However, allopurinol generates superoxide (Berry and Hare, 2004) and some people develop rash as they are allergic to allopurinol (Wallach, 1998; Kong *et al*., 2000). Severe reactions also occur including liver function abnormalities (Fields *et al*., 1996), a fatal complication known as "allopurinol hypersensitivity syndrome" (Umpie´rrez *et al*., 1998; Kong *et al*., 2000) and adverse drug reactions such as Toxic Epidermal Necrolysis syndrome (TENS) (Pacher *et al*., 2006).

The appropriate use of botanical plants to treat various diseases are gaining new interest (Unno *et al*., 2004) and the focus on plant research has increased around the world (Tapsell *et al*., 2006; Triggiani *et al*., 2006). Malaysia houses more than 8,000 species of flowering plants, including shrubs, herbs, lianas, and epiphytes. Some tropical plants and their phytochemicals are worth to be explored as potential XOI as they are already used as food or food supplements for many years and found safe for human bodies (Abd Aziz *et al*., 2011). Polyphenols (Costantino *et al*., 1992), flavonoids (Chang *et al*., 1993; Selloum *et al*., 2001), coumarins (Chang and Chiang, 1995), ellagic acid, valoneic acid dilactone (VAD) (Unno *et al*., 2004) have been reported to be potent plant-based XOI.

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Therefore, this research aims to evaluate XO inhibitory activity from different parts of *Averrhoa carambola, Carica papaya, Dimocarpus longan malesianus, Manilkara zapota* and *Salacca zalacca* so as to discover a natural substitute of plant origin, which could have a superior effect of inhibiting XO activity and can be used as an alternative to allopurinol for the treatment of gout as well as for the treatment of other inflammatory-related diseases. Optimization helped to increase XO inhibitory activity from the selected plant material and potential extraction solvent (data is not included here). This study also emphasized on the efficacy of the optimized extract of *Carica papaya* leaves to inhibit XO, which was compared to allopurinol (synthetic XOI) based on IC_{50} concentrations.

Materials and Methods

Chemicals and reagents

Allopurinol, xanthine and xanthine oxidase (buttermilk) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Dimethylsulphoxide (DMSO), hydrochloric acid (HCl), absolute ethanol, methanol and other reagents of analytical grade were obtained from Merck (Darmstadt, FR, Germany). Potassium di-hydrogen phosphate (KH_2PO_4) and di-potassium hydrogen phosphate (K_2HPO_4) were of the highest purity.

Plant materials

Twenty parts of plants from the selected five plant species were collected fresh in the state of Selangor, Malaysia from June to July 2009. The plant materials were authenticated by the Department of Biotechnology Engineering, Faculty of Engineering, IIUM, Malaysia. The plant species under evaluation were *Averrhoa carambola* (flowers, seeds leaves and ripe fruit peels), *Carica papaya* (leaves, petioles, seeds, unripe fruits, unripe fruit peels and flowers), *Dimocarpus longan malesianus* (ripe fruit peels and leaves), *Manilkara zapota* (leaves, peels and seeds) and *Salacca zalacca* (leaves, petioles, ripe fruit peels, pulps and seeds).

Preparation of crude extracts

The different parts of each plant were washed and oven-dried for 72 h at 40°C. The dried plant materials were grounded using domestic blender to small particle size and stored in a –20°C freezer prior to extraction process. All plant materials were subjected to a standard procedure of solvent extraction process (Harborne, 1998). One gram of each of the dried-powdered plant material was added into 10 ml of extraction solvent and all experiments were conducted in triplicate. Three extraction solvents were employed, namely, 70% methanol, absolute ethanol and distilled water. The mixture of the ground sample and solvent were capped with aluminum foil, and placed in an incubator shaker. The agitation speed of the incubator shaker was set at 100 rpm and ran for 16 h at 30°C. Each mixture of plant material and extraction solvent was filtered using Whatman No. 1 filter paper and the filtrate was collected, concentrated by vacuum rotary evaporator and dissolved in DMSO (100%). Then, it was subjected to XO inhibitory activity assay spectrophotometrically at 295 nm to determine the XOI properties.

Xanthine oxidase inhibitory activity assay

The inhibitory effect on XO was measured spectrophotometrically at 295 nm under aerobic condition, with some modifications, following the method reported by Unno *et al*. (2004) and Umamaheswari *et al*. (2007). A well known XOI, allopurinol (100 µg/ml) was used as a positive control for the inhibition test. The reaction mixture consisted of 300 µl of 50 mM sodium phosphate buffer (pH 7.5), 100 µl of sample solution dissolved in distilled water or DMSO, 100 µl of freshly prepared enzyme solution (0.2 units/ml of xanthine oxidase in phosphate buffer) and 100 µl of distilled water. The assay mixture was pre-incubated at 37°C for 15 min. Then, 200 µl of substrate solution (0.15 mM of xanthine) was added into the mixture. The mixture was incubated at 37°C for 30 min. Next, the reaction was stopped with the addition of 200 µl of 0.5 M HCl. The absorbance was measured using UV/VIS spectrophotometer against a blank prepared in the same way but the enzyme solution was replaced with the phosphate buffer. Another reaction mixture was prepared (control) having 100 µl of DMSO instead of test compounds in order to have maximum uric acid formation.

The equation reported by Naseem *et al*. (2006) was used to evaluate the degree of XO inhibitory activity. Thus, XOI activity was calculated using Eq. 1, in which α is the activity of XO without test extract and β is the activity of XO with test extract.

$$\% \text{ XO inhibition} = (1 - \beta/\alpha) \times 100 \quad (1)$$

Statistical analysis

Statistical analysis was carried out to work out

mean values and standard deviations (mean ± S.D.) from triplicate measurements using Microsoft Office Excel 2007, also used to evaluate the IC_{50} value using dose-response data of logarithmic function curve.

Evaluation on the IC_{50} concentrations

Various concentrations of allopurinol (100, 75, 50, 25, 10, 5, 0.5, 0.1 and 0.05 µg/ml) as well as optimized extract were evaluated for XO inhibitory activity. The dose-response logarithmic function curve was utilized to generate the equation used to calculate the 50% inhibition.

Results and Discussion

Screening of plant materials for XO inhibitory activity

The evaluation of XO inhibitory activity of different parts of *Averrhoa carambola, Carica papaya, Dimocarpus longan malesianus, Manilkara zapota* and *Salacca zalacca* extracts was conducted at a concentration of 100 µg/ml, at which 85% of the crude extracts were found to have XO inhibitory activity. This justified the fact that Malaysian medicinal plants have well diverse chemical structures from their secondary metabolite and chemical diversity (Abd Aziz *et al*., 2011) which makes them promising remedies for gouty ailments in humans. Among them, 23.33% showed greater than 50% inhibition.

The effectiveness of selected extraction solvents to extract bioactive compounds responsible for XO inhibitory activity was studied. The percentages of XO inhibitory activity of all crude extracts obtained by using distilled water were tabulated in Table 1, 70% methanol in Table 2 and absolute ethanol in Table 3. The comparison was also made between the plant extracts in all three extraction solvents and the positive control (allopurinol), to determine the best extraction solvent.

Table 1. Comparison of XO inhibitory activity of crude extracts of twenty parts of plant from five plant species using distilled water as the extraction solvent.

Plant Species	Parts of Plant	XO inhibitory activity (%)[a]
Averrhoa carambola	flowers	0.19 ± 2.4
	seeds	0.00
	leaves	9.34 ± 1.19
	ripe fruit peels	1.47 ± 0.3
Carica papaya	leaves	75.68 ± 0.1
	petioles	0.45 ± 0.4
	seeds	18.92 ± 0.5
	unripe fruits	60.36 ± 0.2
	unripe fruit peels	79.28 ± 0.2
	flowers	17.52 ± 1.6
Dimocarpus longan malesianus	ripe fruit peels	3.59 ± 2.1
	leaves	15.77 ± 1.6
Manilkara zapota	leaves	54.97 ± 0.4
	peels	12.64 ± 0.7
	seeds	2.03 ± 1.5
Salacca zalacca	leaves	2.88 ± 3.6
	petioles	0.00
	ripe fruit peels	4.69 ± 0.9
	pulps	0.51 ± 1.24
	seeds	0.00
Positive control[b]		93.69 ± 0.2
Negative control[c]		0.00

[a] XO inhibitory activity (%) is based on triplicate measurements from a single batch. Results are expressed as means ± SD (n = 3). 20.0% of the distilled water extracts have shown more than 50% XO inhibitory activity.
[b] Allopurinol as the positive control.
[c] Distilled water as the negative control.

Table 2. Comparison of xanthine oxidase inhibitory activity of crude extracts of twenty parts of plant from five plant species using 70% methanol as the extraction solvent.

Plant Species	Parts of Plant	XO inhibitory activity (%)[a]
Averrhoa carambola	flowers	2.46 ± 0.6
	seeds	0.00
	leaves	20.73 ± 0.7
	ripe fruit peels	6.89 ± 2.3
Carica papaya	leaves	79.28 ± 0.3
	petioles	18.02 ± 0.1
	seeds	15.31 ± 0.2
	unripe fruits	64.41 ± 0.2
	unripe fruit peels	72.52 ± 0.1
	flowers	57.91± 0.9
Dimocarpus longan malesianus	ripe fruit peels	10.85 ± 0.1
	leaves	39.42 ± 0.3
Manilkara zapota	leaves	73.04 ± 2.7
	peels	47.33 ± 1.6
	seeds	17.19 ± 1.2
Salacca zalacca	leaves	17.92 ± 0.7
	petioles	0.00
	ripe fruit peels	13.04 ± 0.8
	pulps	2.08 ± 1.3
	seeds	0.00
Positive control[b]		93.69 ± 0.2
Negative control[c]		0.00

[a]XO inhibitory activity (%) is based on triplicate measurements from a single batch. Results are expressed as means ± SD (n = 3). 25.0% of the 70% methanol extracts have shown more than 50% XO inhibitory activity. [b] Allopurinol as the positive control.
[c] 70% methanol as the negative control.

Table 3. Comparison of XO inhibitory activity of crude extracts of twenty parts of plant from five plant species using absolute ethanol as the extraction solvent.

Plant Species	Parts of Plant	XO inhibitory activity (%)[a]
Averrhoa carambola	flowers	2.47 ± 0.4
	seeds	0.00
	leaves	23.61 ± 0.8
	ripe fruit peels	7.11 ± 0.9
Carica papaya	leaves	78.38 ± 0.1
	petioles	8.11 ± 0.1
	seeds	19.82 ± 0.1
	unripe fruits	68.47 ± 0.6
	unripe fruit peels	71.17 ± 0.3
	flowers	66.03 ± 0.5
Dimocarpus longan malesianus	ripe fruit peels	13.41 ± 1.42
	leaves	46.88 ± 1.7
Manilkara zapota	leaves	70.81 ± 0.2
	peels	41.03 ± 0.1
	seeds	11.81 ± 2.4
Salacca zalacca	leaves	19.66 ± 1.02
	petioles	0.00
	ripe fruit peels	12.85 ± 0.5
	pulps	2.88 ± 0.5
	seeds	0.00
Positive control[b]		93.69 ± 0.2
Negative control[c]		0.00

[a]XO inhibitory activity (%) is based on triplicate measurements from a single batch. Results are expressed as means ± SD (n = 3). 25.0% of the ethanol extracts have shown more than 50% XO inhibitory activity. [b] Allopurinol as the positive control. [c] Absolute ethanol as the negative control.

The highest XOI activity was shown by distilled water extract of *Carica papaya* unripe fruit peels and 70% methanolic extract of *Carica papaya* leaves with 79.28 ± 0.2% and 79.28 ± 0.3%, respectively, followed by absolute ethanolic extract of *Carica papaya* leaves with 78.38 ± 0.1% and distilled water extract of *Carica papaya* leaves with 75.68 ± 0.1%. Seeds of *Averrhoa carambola* and *Salacca zalacca*, and petioles of *Salacca zalacca* have shown no inhibition activity for all extraction solvents used. These parts of plant from other plant species have also demonstrated the least XO inhibitory activity probably due to limited bioactive compounds present. *Carica papaya*'s leaves and unripe fruit peels have demonstrated the best source of raw material for obtaining the XOI compound as each exhibits more than 70% inhibition of XO under all three extraction solvents. In fact, leaves of all plants under evaluation have shown considerable activity for XO inhibition, substantiate the fact that secondary metabolites in the leaves contain diverse classes of bioactive phenolic compounds such as polyphenols, tocopherols and alkaloids (Hismath *et al*., 2011), which may act as XOI.

Selection of plant material for optimization and further investigation on its superiority to inhibit XO

Selection of plant sample for optimization depended upon the issue of availability and sustainability. Malaysian fruits are mostly seasonal and not available throughout the year. In addition, some of the trees take years to grow, which can also position the production of new XOI at risk. Of all the plant species under evaluation, *Carica papaya* has shown promising future to be utilized as new natural source of XOI. Moreover, *Carica papaya* is not fastidious (Chan, 2009) and can be found in almost every corner of Malaysia. In addition, Malaysia was also ranked as the second most important exporter of *Carica papaya* in the world in 2004 with a total volume of 58, 149 million tonnes accounting for 21% of the global trade (Chan, 2009). Apart from the main uses of *Carica papaya* as fresh fruit and for the production of drinks, certain parts of this plant have been developed for usage in medicine and cosmetic industry due to its vast chemical compounds found. The papain, which is a proteolytic enzyme that breaks and degrades protein, is the substance that gives the plant medicinal and commercial value. In pharmaceutics, papain is used for suppression of inflammation, treatment of gangrenous wounds and for various ailments (Chan, 2009). Papaya leaves contain the bitter alkaloids, carpaine and pseudocarpaine, which act on the heart and respiration (Perry and Metzger, 1980).

Although the activity obtained by the leaves of *Carica papaya* in distilled water was slightly lower than other parts and solvents, but it was selected for optimization due to the fact that fruit peels are not economical, especially for large scale production. Furthermore, leaves were available throughout the year, relatively cheap, accessible and easy to manage.

Investigation on the efficacy of extraction solvents under evaluation to extract XOI compound

Both methanol and ethanol have shown better capacity to extract XOI from all parts of plants as 25% of all plant extracts have demonstrated more than 50% inhibition under these two solvents. On the other hand, only 20% of all plant extracts using distilled water as the extraction solvent have shown more than 50% XO inhibitory activity.

Although methanol and ethanol have shown better characteristic in obtaining high percentage of XOI activity, probably due to the nature of biological active components (alkaloids, flavonoids, essential oil, terpenoids, etc), which may be enhanced in their presence and the stronger extraction capacity

may have produced a greater number of active constituents responsible for XOI activity (Ghosh *et al*., 2007) but scalling-up requires large quantities of solvent to be used and it may be necessary to use the most economical solvent that fulfils the extraction and safety criteria. In addition, alcohol was known as nervous system depressant. They impair the transmission of nerve signals, ultimately leading to a block of respiration (Bailey Jr. and Bailey, 2000). Methanol is considered highly poisonous solvent as it can upset the acid-base balance of the body. Thus, less toxic solvents with similar capability to enhance or produce the desired activity must be employed wherever possible. Moreover, there are strict laws governing the levels of solvent residues in extracts for food or drug used especially for oral consumption; the permitted limits vary depending on the toxic potential of the residues.

Even though there are numerous conventional extraction techniques based on organic solvents (i.e. methanol and ethanol) have been applied to the extraction of XOI from medicinal plants (Owen and Johns, 1999; Ferraz Filha *et al*., 2006; Garrido *et al*., 2008), these methods result to undesirable effects on the environment and on food components. Water is essential for all living things and often referred to as a universal solvent because many substances dissolve in it. Unlike methanol and ethanol, water is readily available, practically applicable and relatively cheap. The partial charge that develops across the water molecule grants its unique dissolving properties hence helps make it an excellent extraction solvent (Rovio *et al*., 1999; Carpi, 2005). In fact, many have applied aqueous medium for the extraction of XOI (Unno *et al*., 2004; Umamaheswari *et al*., 2007).

Thus, further development of process conditions for extraction using distilled water and leaves of *Carica papaya* was carried out in order to formulate a promising remedy for the treatment of gout by maximizing the inhibition of XO, subsequently reducing uric acid formation that leads to development of gout.

Comparison of XO inhibitory activity between allopurinol and optimized extract

The XO inhibitory activity of allopurinol and optimized distilled water extract of *Carica papaya* leaves at various concentrations was represented in Table 4. Each has demonstrated more than 50% XO inhibition starting from a concentration of 5 µg/ml. The highest residual or percentage difference between allopurinol and the optimized sample was at 50.00 µg/ml with 8.72%, followed by 75.00 µg/ml with 8.26% and 25.00 µg/ml with 7.83%. The smallest percentage difference was at 0.05 µg/ml with only 4.72%. Overall, these small percentages are encouraging for *Carica papaya* leaves to be utilized for large-scale purposes for the production of anti-gout supplement, an alternative to allopurinol. In folk medicine, *Carica papaya* has been used as an important traditional herbal medicine due to its vast bioactive compounds found including kaempferol, quercetin, 5, 7-dimethoxycoumarin, alkaloids, carpaine and pseudocarpaine. Any of these bioactive compounds may contribute to XO inhibitory activity as they have previously received considerable attention because of their physiological functions such as antioxidant, antimutagenic and antitumor activities (Othman *et al*., 2007; Charoensiddhi and Anprung, 2008; Ruzaidi *et al*., 2008; Ayub *et al*., 2010).

Table 4. XO inhibitory activity of allopurinol and optimized distilled water extract of Carica papaya leaves at various concentrations.

Concentration (µg/ml)	XO inhibitory activity (%) of allopurinol[a]	XO inhibitory activity (%) of optimized distilled water extract of *Carica papaya* leaves[b]
100	93.69 ± 0.2	86.93 ± 1.9
75	89.37 ± 0.5	81.11 ± 2.5
50	81.54 ± 3.07	72.82 ± 1.51
25	74.12 ± 0.74	66.29 ± 3.03
10	66.69 ± 2.78	59.56 ± 0.5
5	62.94 ± 2.44	56.33 ± 0.9
0.5	47.93 ± 1.29	42.08 ± 0.2
0.1	39.62 ± 1.45	33.97 ± 1.05
0.05	22.38 ± 3.14	17.66 ± 3.62

Results are expressed as means ± SD (n=3). [a]Synthetic XOI (positive control).[b]Distilled water extract of Carica papaya leaves produced at optimum process conditions for extraction.

IC_{50} evaluation of the optimized extract against allopurinol on XO inhibitory activity

XO inhibitory activity for both allopurinol and optimized extract of *Carica papaya* leaves were also expressed in term of IC_{50}, the concentration of positive control and optimized sample needed to achieve 50% inhibition of XO under experimental conditions. The IC_{50} value was calculated using dose-response logarithmic function curve (Allopurinol, $y_1 = 41.08 \ln(x) - 4.233$, $R^2 = 0.990$; Optimized extract, $y_2 = 37.95 \ln(x) - 5.655$, $R^2 = 0.983$). In this study (Figure 1), it was found that the allopurinol showed lower IC_{50} value (3.74 µg/ml) as compared to the optimized sample (4.33 µg/ml).

Although by evaluating on the IC_{50} concentration, allopurinol is still the best XOI for the treatment of gout and other related-inflammatory diseases as compared to the optimized extract but the difference in both IC_{50} concentrations is small, only 0.59 µg/ml. Following many of the side effects caused by

allopurinol, it is better to utilize natural XOI instead. The strong inhibition of XO activity exhibited by allopurinol justified the relevant of its usage for clinical management of gout and related-inflammatory diseases (Pacher *et al*., 2006). Meanwhile, the bioactive substances present in the extracts could be a possible mechanism for its XO inhibition activity. These findings are encouraging to plan clinical studies in hyperuricemic patients.

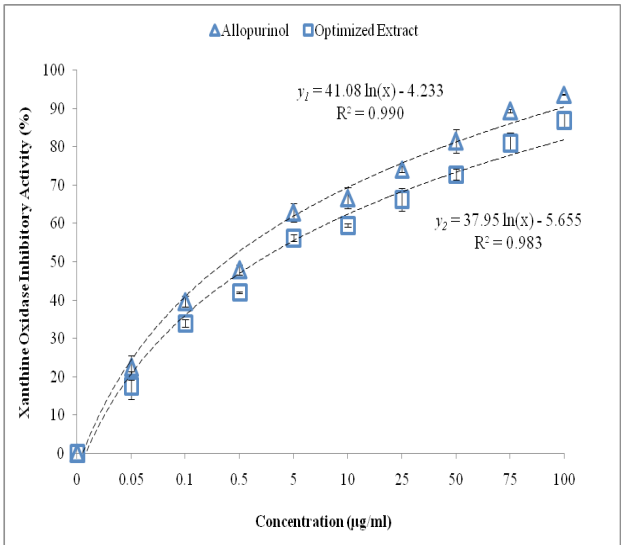

Figure 1. XO inhibitory activity (%) as a function of allopurinol and optimized extract concentrations. y_1 represents dose-response logarithmic function curve of allopurinol (R^2 = 0.990) and y_2 represents dose-response logarithmic function curve of optimized extract (R^2 = 0.983). Results are expressed as means ± SD (n = 3). IC_{50} value for allopurinol (3.74 µg/ml) is lower than the optimized sample (4.33 µg/ml).

Conclusion

All plant species under investigation exhibit XO inhibitory activity but not all parts of plant have shown the same characteristic. Distilled water extract of *Carica papaya* leaves have shown promising XO inhibitory activity as compared to the seeds, petioles, flowers, unripe fruits and unripe fruit peel. The percentage of XO inhibition of this respective sample (distilled water: *Carica papaya* leaves) of 86.93 ± 1.9% obtained by extracting at the developed process conditions after optimization study have shown promising future to be developed and formulated as anti-gout supplement with only 6.76% difference in the XO inhibition studies as compared to the synthetic XOI, allopurinol (93.69 ± 0.2%). Furthermore, The IC_{50} concentration for allopurinol is 3.74 µg/ml, whereas, optimized extract exhibits IC_{50} of 4.33 µg/ml. The efficacy of distilled water as the extraction solvents was commendable because most of the plants used to treat gout were administered as decoctions and infusions, so the biologically active compounds were most likely water-soluble. In addition, the possibility of any harmful residue due to use of organic solvent was also avoided.

The selection of tropical plants used in ethno-medicine and screening of their extracts for pharmacological activity may provide identification of newer medicaments for the treatment of various ailments especially gout. Further research on *Carica papaya* leaves, for improving the existing method or identifying the active constituents that exhibit a significant XO inhibitory activity, warranted more attention as anti-gout compound from natural sources could be marketed in large scale as a substitute to the current irresponsive synthetic medicine.

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