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EFFECT OF SOME PLANT EXTRACTS ON SERUM URIC ACID LEVELS, AND XANTHINE OXIDASE ACTIVITY IN VITRO AND IN OXONATE-INDUCED HYPERURICEMIC RATS

***Hasan F. Al-Azzawie and Samah A. Abd**

Dept. of Biotechnology/College of Science/Baghdad University/Iraq.

***Correspondence for Author: Hasan F. Al-Azzawie**
Dept. of Biotechnology/College of Science/Baghdad University/Iraq.

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ABSTRACT

Increased serum uric acid is known to be a major risk related to the development of several oxidative stress diseases. The present study sought to investigate the inhibitory effect of three aqueous plant extract: Olea europaea (Olive), Zingiber officinale (Ginger) and *Brassica oleracea* (Red cabbage) on Xanthine Oxidase enzyme (XO), the key enzyme involved in purine degradation and uric acid production. The active compounds in extracts were investigated using phytochemical analysis methods which observed that all plant extracts were rich in active compounds, particularly phenolic, flavonoids. Olive leaf extract (OLE), Red cabbage extract (RCE) and Ginger extract (GE) were screened according to their inhibitory effect against xanthine oxidase enzyme *in vitro*. Results observed that the highest inhibitions were obtained with GE (87.97± 1.45%) followed by OLE and RCE (80.00 ± 2.20 and 53.72 ± 1.76% respectively) compared with standard drug allopurinol (93.00 ± 0.57%). *In vivo* inhibitory activity of GE at different doses was evaluated in potassium oxonate induced rats (Hyperuricemia model) and in normal healthy rats. Oral administration of ginger extract with (100 and 250 mg/ kg) once a day for 4 weeks to the hyperuricemia rats groups significantly (P<0.05) reduce the mean serum uric acid levels and inhibited XO activity , while GE did not have a significant effect in normal rats groups.

KEYWORDS: Hyperuricaemia, Xanthine Oxidase, Uric Acid, Ginger, Red Cabbage, Olive Flavonoids.

INTRODUCTION

Hyperuricemia which is recognized by irregular elevated in plasma uric acid concentration is emerge to be expanded worldwide.[1] However, more recent investigations have shown by providing compelling evidence to support the association between hyperuricemia and increased risk of oxidative stress diseases such as cancer, diabetes, hypertension, kidney disease and cardiovascular disorders.[2, 3, 4, 5] The regulation of uric acid production has been considered as a vital element in the prevention and treatment of hyperuricemia.[6] Liver xanthine Oxidoreductase is the key enzyme catalyses the oxidation of hypoxanthine to xanthine from purine catabolism to uric acid. This enzyme exists in two definite forms. Under certain physiological conditions Xanthine dehydrogenase (XDH) is the conventional active form, in synchronization to the degradation of (adenine tri phosphate) ATP into adenine and xanthine, an excessive transformation of XDH to xanthine oxidase (XO) takes place. The latter Employs molecular oxygen as electron acceptor and induce the formation of reactive oxygen species (ROS) in synchronization with uric acid production.[7] Therefore, the inhibition of XO activity subsequently reduce uric acid and ROS production and leads to anti-hyperuricemic and antioxidative effects.

Allopurinol is the only clinically used as Xanthine oxidase inhibitor and has served as a prevalent uric acid-reducing agent in the past four decade.[8, 9] However, some severe counteractive effects like nephropathy, allergic reactions and hepatitis limit the clinical use of allopurinol.[10] Various *in-vitro* investigation approved the XO inhibitory activity of some phytochemicals such as flavonoids.[11, 12, 13, 14] A promising approach for hyperuricemia and its complications might be a alternative therapy using dietary flavonoids and hypouricemic curative at a suboptimal dosage which are devoid of the undesirable side effects of Allopurinol. The present study was designed to screen natural compounds for their xanthine oxidase inhibitory potential. Xanthine oxidase inhibitors (XOI) are used in the treatment of gout. On the basis of reported literature the present work was carried out to screen ginger, red cabbage and olive leaf extracts in the search of new less contraindicative XO inhibitors. The aim of this research was to determine the effects of plant extracts on serum uric acid levels and xanthine oxidase activity.

MATERIALS AND METHODS

Plant material

Fresh Samples of red cabbage plant and ginger were purchased from Iraqi local market in Baghdad, Olive leaves were obtained from handpicked. The samples oven-dried for 96 h at 40°C. Dried plant materials were grounded using domestic blender to small particle size stored in a dry place in the dark for the experimental study.

Aqueous extraction

All plant materials were subjected to a standard procedure of solvent extraction process. 250 g of each of the dried powdered plant material was added into 750 ml of 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. The 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.

Preliminary Phytochemical screening

The crude plant extracts were screened to detect the presence or absence the secondary metabolites such as alkaloids, steroidal compounds, phenolic compounds, flavonoids, saponins, tannins and anthraquinones according to standard procedures.[15]

Test for alkaloids

100 mg of crude extracts was dissolved individually in 5ml of 1% hydrochloric acid, filtered and tested with Dragendroff's reagent and Mayer's reagent separately. Any precipitate or turbidity with the reagents suggests the presence of alkaloids.

Test for flavonoids

Five milliliters of ethyl acetate was added to a solution of 0.5 g of the extract in water. The mixture was shaken, allowed to settle and inspected for the production of yellow color in the organic layer which is taken as positive for flavonoids.

Test for phenolic compounds

100 mg of the dried extract was dissolved in water. Few crystals of ferric sulfate were added to the mixture. Formation of dark violet color indicated the presence of phenolic compounds.

Test for saponins

0.5 g of the dried extract was dissolved in 10 ml of distilled water in a test tube. The test tube was stoppered and shaken vigorously for about 30 seconds. The test tube was allowed to stand in a vertical position and observed over a 30 minute period of time. If a "honey comb" froth above the surface of liquid persists after 30 min. the sample is suspected to contain saponins.

Test for Steroids and Terpenoids

0.5 g of the dried extract was dissolved in 1ml of chloroform and filtered. 1 ml of acetic acid was added to the filtrate and then a few drops of conc. sulphuric acid were run down the side of the test tube. The appearance of a pink or pinkish-brown ring / color indicates the presence of terpenoids. The appearance of blue, bluish-green or a rapid change from pink to blue colors indicates the presence of steroids and a combination of pink and these colors indicates the presence of both steroids & terpenoids.

Test for tannins

0.5 g of the dried extract was dissolved in water. The solution was clarified by filtration. 10% ferric chloride solution was added to the clear filtrate. This was observed for a change in color to bluish black.

Test for Carbohydrates and glycosides

0.5 g of dried extract was dissolved in 4 ml of distilled water and filtered. The filtrate was subjected to the following tests to detect the presence of carbohydrates and glycosides.

Molisch's test

The filtrate was treated with 2-3 drops of 1% alcoholic ∝-naphthol and 2ml of concentrated sulphuric acid was added along the sides of the test tube. Appearance of brown ring at the junction of two liquids shows the presence of carbohydrates. Another portion of extract was hydrolyzed with dilute hydrochloric acid for few hours on a water bath and hydrolysate was subjected to Legal's test to detect the presence of glycosides.

Legal's test

To the hydrolysate 1ml of pyridine and few drops of sodium nitroprusside solution were added and then it was made alkaline with sodium hydroxide solution. Appearance of pink to red color shows the presence of glycosides.

Animal model of hyperuricemia in rats

Potassium oxonate the uricase inhibitor was used to induce experimentally-hyperuricemia in rats to study antihyperuricemic and antioxidant effects of plant extracts according to the method reported by Hall *et al.*, (1990).[16] Briefly, 250 mg/kg potassium oxonate (PO) dissolved in 0.8% carboxymethyl cellulose sodium salt (CMC-Na) was administrated intraperitoneally 1 hour before oral administration of test compounds to increase serum urate levels. Allopurinol used as a positive control, was prepared in 0.9% saline. All freshly prepared ginger extract were administrated to the corresponding groups by oral gavages once a day for 4 weeks.

Experimental design to study in vivo inhibition of XO activity

The animals were randomly divided into ten equal groups (n = 6). group 1: untreated, nonhyperuricemic

animals received only carboxy methyl cellulose- Na (CMC-Na) as vehicle.; group 2: normal animals given 50 mg/kg GE; group 3: normal animals given 100 mg/kg GE; group 4: normal animals given 250 mg/kg GE; group 5: normal animals given 5mg/kg allopurinol; group 6: hyperuricemic animals; group 7: hyperuricemic animals given 50 mg/kg GE; group 8: hyperuricemic animals given 100 mg/kg GE; group 9: hyperuricemic animals given 250 mg/kg GE; group 10: hyperuricemic animals given 5mg/kg allopurinol. The ginger extract was administered to the corresponding groups by oral gavages once a day for 28 days. (Note. PO at the same dose was administrated intraperitoneally 1 hour before the oral administration of ginger extract to each animal in group 7, 8 and 9.

Sample preparation

At the end of the experiment, rats were sacrificed and the blood collected by transcardiac puncture 1 hour after administration of the test compound. Serum was obtained by centrifuging blood sample at 6000 rpm for 10 minutes, the serum stored at -20 °C until used.

Determination of serum uric acid level

About 20 µl of serum was added to a test tube containing 1 ml of reagent mixture was kept aside for 5 min at 37ºC and the absorbance was taken against the regent blank at 546 nm. Absorbance of the standard was taken by using uric acid standard (6mg/dl) as mentioned above. Concentration of uric acid was determined as follows:

Determination of serum XO Activity

$$\text{Uric Acid concentration. (mg/dl)} = \frac{\text{Absorbance of standard}}{\text{Absorbance of sample}} \times 6$$

Serum XO activity (cytoplasmic xanthine oxidase (EC1.17.3.2)) was measured according to the method of Prajda and Weber, where activity is measured by determination of uric acid from xanthine.[17] Serum was incubated for 30 min at 37°C in phosphate buffer (pH 7.5, 50 mM) containing xanthine (4 mM). The reaction was stopped adding 20µL 100% TCA. The mixture was then centrifuged at 4000 ×g for 20 min. Uric acid was determined in the supernatant by absorbance at 292 nm against a blank. The results are expressed as units per milliliter (U/mL).

RESULTS

Phytochemical screening was done using color forming and precipitating chemical reagents on the dried extract of olive leaf, red cabbage and ginger rhizomes. Results obtained from the tests were summarized in Table (1). It was shown that olive leaf extract (OLE) contains: alkaloids, steroids, terpenoids, phenplic compounds, flavonoids, charbohydrate and glucosides. while the red cabbage extract (RCE) contains: phenolic compounds, flavonoids, charbohydrate and glucosides, and the ginger extract (GE) contains: alkaloids, terpenoids, phenolic compounds, flavonoids, saponin and glucosides.

Table 1: Results of Phytochemical screening of the three plant extracts.

Phytochemicals tests	OLE	RCE	GE
Alkaloids	+	-	+
Steroids	+	-	-
Terpenoids	+	-	+
Phenolic compounds	+	+	+
Flavonoids	+	+	+
Saponins	-	-	+
Tannins	-	-	-
Carbohydrate	+	+	-
Glycosides	+	+	+

Screening the plant extracts inhibitory effect against xanthine oxidase enzyme in vitro

Each of the crude extracts OLE, RCE and GE elicited a dose- dependent inhibition of xanthine oxidase resulted in a decreased production of uric acid which was measured spectrophotometrically in order to choose the extract with the higher inhibition effect for later *in vivo* studies. IC_{50} values were obtained through the slope of the plot of concentrations used (50, 150, 250 mg/ml) against percent inhibition determined at each concentration. All of the 3 extracts assayed demonstrated XOI activity greater than 50% at a concentration of 250mg/ml. Table 3-4 showed that GE has the highest percent inhibition 87.97 ± 1.45 % at the concentration 250 mg/ml, followed by OLE 80.00 ± 2.20 % and finally RCE has the lowest inhibition percent 53.72 ± 1.76% at the same concentration used. The high concentration of GE (250 mg/ml) did not show a significant difference with allopurinol for this reason it has been chosen for the *in vivo* studies.

Table 2: Inhibitory effect of crud extracts at different concentration and the IC_{50} for each extract (Values are mean ± SEM).

Extract	Inhibition %			IC_{50} mg/ml
	50 mg/ml	150 mg/ml	250 mg/ml	
OLE	11.47 ± 1.86	34.01 ± 0.98	80.00 ± 2.20	114.02
RCE	33.67 ± 2.02	61.33 ± 1.85	53.72 ± 1.76	230.15
GE	35.32 ± 3.84	66.69 ± 1.45	87.97 ± 1.45	99.37
Allopurinol	93.00 ± 0.577			

Effect of crud ginger extract on serum uric acid levels and XO inhibition *In vivo*

A time dependent study on ginger extract were conducted to evaluate the effect of GE *in vivo* on serum uric acid level during 28 day of the oral administration of GE and allopurinol in hyperuricemia model and normal healthy rats. The results (Table 3) show that administration of crud ginger extract did not cause any significant effect in serum uric acid in normal rats after 1, 14, 28 days, but allopurinol, as a putative inhibitor of XO, significantly reduced (P<0.001) the levels of serum uric acid in the normal rats from the first day. In the hyperuricemic groups treated with ginger extract (100 and 250 µg/ kg) the uric acid levels significantly (P<0.001) reduced compared to hyperuricemic control rats after 14 day of treatment. The uricase inhibitor, potassium oxonate resulted in significant (P<0.001) hyperuricaemia in rats, as indicated by an increase in the serum urate levels that reaches to 3.85 ± 0.91 when compared to the control group (normal) serum uric acid 2.25 ± 0.07. The standard drug Allopurinol at a dose of 5 mg/kg elicited significant reduction of serum urate level compared to hyperuricaemic rats, the serum uric level was lowered down by 52.15% as compared to the hyperuricemic control group. High concentration group 250 mg/kg of GE show the highest reduction activity, It significantly reduced the urate levels in hyperuricemic rats to 2.301 ± 0.175 the serum uric and levels of rats was lowered down by 40.38% as compared to the hyperuricemic control group, followed by medium concentration group 100 mg/kg of GE significantly lowered the urate levels in hyperuricemic rats to 2.97 ± 0.06 the serum uric and levels of rats was lowered down by 22.89 % as compared to the hyperuricemic control group. Low concentration group 50 mg/kg of GE did not produce any significant reduction in serum uric acid levels when compared to the hyperuricemic group. The results showed that ginger extract and allopurinol exert their hypouricemic effects in a time-dependent manner.

Table 3: Effect of the orally intake of crud ginger extract on serum uric acid levels (Mean ± SE mg/dl) in normal and hyperuricemia rats induced by potassium oxonate (a time dependent study).

Group	Day1	Day14	Day 28
Normal (vehicle)	2.00±0.33	2.21±0.24	2.25 ± 0.07
Normal + GE (50 mg/kg)	2.21 ±0.29	2.22±0.36	2.23± 1.67
Normal + GE (100 mg/kg)	2.17±0.30	2.24±0.26	2.21± 0.45
Normal + GE (250 mg/kg)	2.22±0.28	2.20±0.25	2.17± 0.33
Normal + Allopurinol (5 mg/kg)	$1.68±0.27^{###}$	$1.62±0.29^{###}$	$1.24 ± 0.86^{###}$
Hyperuricemia	$3.66±0.48^{###}$	$3.56±0.47^{###}$	$3.85 ± 0.91^{###}$
Hyperuricemia+GE (50 mg/kg)	3.64±0.45	3.48±0.36	3.67 ± 0.17
Hyperuricemia +GE (100 mg/kg)	3.57±0.43	3.31±0.32**	3.17 ± 0.06***
Hyperuricemia+GE (250 mg/kg)	3.56±0.38	2.12±0.29***	2.30 ± 0.15***
Hyperuricemia+ Allopurinol(5 mg/kg)	2.35±0.28***	1.97±0.38***	1.84 ± 0.11***

*P < 0.05 when compared with hyperuricemia control group, **P < 0.01 when compared with hyperuricemia control group, ***P < 0.001 when compared with hyperuricemia control group, $^{###}P$ < 0.001 when compared with vehicle control group.

Evaluation the effect of GE on serum xanthine oxidase activity were done in a dose dependent study, results (**Table 4)** show that in normal groups treated with GE did not make a significant effect on XO activity, but Allopurinol did. In hyperuricemia groups treated with flavonoids (250,100 and 50mg/ kg.b.w) there were a significant reduction in serum XO activity by 57.14%, 40.10 % and 12.08 % respectively.

Tabl 4: Results of orally intake of three different concentration of GE on serum XO activity in normal and hyperuricemia rats induced by potassium oxonate (a dose dependent study).

Group	XO activity (U / ml)	XO inhibition %
Normal (vehicle)	1.42±0.21	-
Normal + GE (50 mg/kg)	1.42±0.13	0.42
Normal + GE (100 mg/kg)	1.41±0.11	0.7
Normal + GE (250 mg/kg)	1.40±0.05	1.40
Normal + Allopurinol	1.04±0.25	26.76
Hyperuricemic	3.64±0.21	-
Hyperuricemic+GE (50 mg/kg)	3..20±0.42	12.08
Hyperuricemic +GE (100 mg/kg)	2.18±0.19	40.10
Hyperuricemic+GE (250 mg/kg)	1.56±0.29	57.14
Hyperuricemic+ Allopurinol	1.13±0.27	68.95

DISCUSSION

Flavonoids and other phenolic are especially common in *olea europaea* leaves, which consider as good inhibitor for XO also has antioxidantive action. The contributing of the anti-gout activity of olive leaves and its basic phenolic compounds has been reported previously by Flemmig.[18] RCE is rich with phenolic acids and Anthocyanins which considar a potent antioxidants, quench free radicals, inhibit XO and terminate the chain reaction that is responsible for the oxidative damage.[19] Many studies revealed that anthocyanins exhibit antioxidant properties and could therefore be useful in the treatment of pathologies where free radical production plays a key role.[20] Anthocyanidins have superior antioxidant activity compared to their respective anthocyanins and the activity decreases as the number of sugar moieties increase.[21] Several studies on ginger showed there are certain active constituents present and antioxidants within ginger extract including flavonoids, these phytochemicals improve total antioxidant capacity, suppress destructive oxygen free radicals and prevents oxidative stress damage.[22, 23] Our study also showed that ginger extract has a higher XOI activity than other studied plants.

Based on the *in vivo* results, the oral administration of GE exerts notable hypouricemic effects in hyperuricemic but not in normal rats. In contrast, allopurinol reduced serum uric acid levels of both normal and hyperuricemic rats and the levels even reached to the level lower than that of normal values. These results indicate that GE might bring fewer side effects than allopurinol in treatment of hyperuricemia. On the other hand, this property of GE could be considered as an advantage for this medicinal plant. Although the elevated levels of uric acid in the circulation could give rise to gout and possibly other pathological conditions, the antioxidant action of uric acid, particularly its ability to inhibit DNA damage, is also well documented.[24] Thus, excessive lowering of the uric acid level in the circulation beyond that of the normal range might even be counterproductive.[24, 25] Kong *et al.* have also shown that the water extract of Ermiao wan (a Chinese herbal medicine used in the treatment of acute gout have less inhibitory effects on serum uric acid levels in normal mice compared with those animals pretreated with potassium oxonate.[26]

Taking into account that GE as a dietary vegetable can be used safely long-term; this feature of GE makes it a possible alternative for allopurinol, or at least in combination therapy to minimize the side-effects of allopurinol. The results also indicate that GE exerts their hypouricemic effects in a time-dependent manner in oxonate-pretreated rats. In GE treated-hyperuricemic rats, uric acid reduction was not statistically significant on the first day, but after 14 and 28 days of intervention, a significant reduction was observed in the uric acid levels of hyperuricemic rats.

Unlike these above data, the hypouricemic effect of allopurinol was statistically significant ($p \leq 0.01$) even after 1 day of the drug administration indicating the quicker onset of allopurinol action compared to that of GE and flavonoids fraction .The inhibitory effect of GE and its major flavoinod Gingerols on serum XO activity was also confirmed in this study. XO is the key enzyme in the catabolism of purines and has a critical role in the endogenous production of uric acid.[27] Several *in-vitro* studies confirmed the XO inhibitory activity of some flavonoids. These compounds are structurally similar to XO substrate and so can inhibit the enzyme activity.[28] Therefore the hyperuricemic property of GE extract, observed in this study, could be explained at least in part by the inhibitory effects of them on XO activity. The extent of reduction in XO activity elicited by allopurinol was much higher than that observed when the GE administration in both normal and hyperuricemic groups.

According to these studies, the involvement of other possible mechanisms such as enhanced uric acid clearance or actions on other purine metabolizing enzymes cannot be ruled out.[26, 29] This could be further supported by the existence of some hypouricemic compounds including natural products that are devoid of XO inhibitory activity.[25, 26, 29] It seems that the inhibitory effects of GE, on XO activity in hyperuricemic rats are more dominant than their effects on the normal activity of the enzyme. Similar results have been reported by Zhao *et al.*[30]

CONCLUSION

All the studied plants showed inhibitory activity against XO enxyme. Ginger are able to reduce uric acid levels in hyperuricemic rats with no effects on the level of this biological metabolite in normal animals. Such hypouricemic effects may be attributed, at least in part, to XO inhibitory action of them. Therefore, the use of suboptimal dosages of allopurinol in combination with Ginger extract intake may provide a safer approach for prevention and treatment of hyperuricemia. Further investigations to explore the effect of other components of Ginger and define their clinical efficacy would be highly desirable.

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