# PHARMACEUTICAL SCIENCES

## Inhibition of Xanthine Oxidase by Puerto Rican Plant Extracts

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***Objectives.*** **This study was conducted to search for xanthine oxidase inhibitors in natural products obtained from plants collected in Puerto Rico and to assess the influence of these extracts in the prevention of cataractogenesis.**

***Background.*** **Allopurinol is currently a xanthine oxidase inhibitor used in the treatment of gout. New alternatives with increased therapeutic activity and less side effects should be investigated. Preclusion of cataractogenesis in diabetic rats is also the focus of this investigation. Natural products in the form of plant extracts from Puerto Rico offer a rich and relatively untapped source for the discovery of new drugs that may address these kind of problems.**

***Methods.*** **Nineteen collections of Myrtaceae plant extracts were screened for xanthine oxidase inhibition. A spectrophotometrical method was used employing allopurinol as positive control and a blank as negative control. A protocol of the assay with slight modifications was followed from the literature. Two extracts with the highest percentages of xanthine oxidase inhibition were evaluated for possible prevention of cataractogenesis in streptozotocin diabetic rats. The animals were given to drink these plant extracts *ad libitum* for three months while controls received water. The appearance of cataracts was assessed physically.**

***Results.*** **Two of the nineteen plant extracts showed high inhibition percentages of xanthine oxidase. *Eucalyptus deglupta* and *Syzygium malaccense* displayed 51% and 64% inhibitions ($IC_{50}$ 44.5 µg/ml and $IC_{50}$ 51 µg/ml), respectively. As for the cataractogenesis inhibition, laboratory animals that drank *E. deglupta* for three months did not develop cataracts.**

***Conclusions.*** **Two plant extracts provided positive results with varying degrees of inhibition of xanthine oxidase. *S. malaccense* demonstrated the greatest xanthine oxidase inhibitory activity whereas *E. deglupta* presented the best finding for cataractogenesis prevention. The procedures used in this investigation are useful for the *in vitro* screening of xanthine oxidase inhibition and the *in vivo* evaluation of cataractogenesis prevention.** *Key words: Xanthine oxidase, Allopurinol, Plant extracts, Cataractogenesis, Puerto Rico.*

It is known that uric acid accumulation occurs in conditions like gout, leukemia, myeloid metaplasia, and polycythemia vera. In gout, a known genetic disease, the accumulation of uric acid in the tissues produces pain in the articulations. There are uricosuric agents such as probenecid, hydrochlorothiazide, and salicilates that have been used to treat excessive uric acid accumulation. Nevertheless, it has been reported that in some cases, patients tend to develop kidney stones. Allopurinol, a xanthine-oxidase (XO) inhibitor, is another alternative in the treatment of uric acid buildup. The formation of kidney stones is commonly avoided with allopurinol chemotherapy. However, a condition known as granuloma annulare was observed in two patients treated with allopurinol. Following its discontinuation, the cutaneous granulomas healed without relapse (1).

The search for new and useful drugs in treating human gout is important and should include the evaluation of our botanical resources. Ethnobotanical data have provided valuable information about the XO inhibitory action of plants. Previous papers have shown a good association between XO inhibitory activity and plant species of the Myrtaceae family (2,3). XO inhibitors have also been sought in plants of different families. As

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examples, Noro et al. isolated several flavonoids from the flowers and buds of *Daphne genkwa* (Thymelaceae). The flavonoids apigenin and luteolin showed particularly strong inhibitory activity (4). In another study, the influence of nineteen flavonoids on XO was investigated by Iio et al. Myricetin, kaempferol, and quercetin strongly inhibited the enzyme at 50 μM (5). A preliminary pharmacological study on *Eugenia uniflora* leaves (Myrtaceae), a Paraguayan folk medicine, demonstrated that the flavonoids quercetrin, quercetin, myricetrin and mirycetin were responsible for the XO inhibitory action of the plant extract (6). Based on these results, screening of XO inhibition by plant extracts of South American Myrtaceae was carried out in Paraguay (2) and Chile (3). Hayashi et al. examined sixty native crude drugs from Paraguay. Bioassayed-directed fractionation of one of the active plants, *Schinus terenbinthifolius* (Anacardiaceae) resulted in the isolation of pentagalloylglucose, a tannic derivative (7). In another study, the stems of *Bouganvillea spectabilis* (Nyctaginaceae) have been used as folk medicine for hepatitis. One of the isolated compounds from the extract of this plant, caffeic acid, was found to be a strong inhibitor of XO ($IC_{50}$ 39 μM) (8). Another investigation with a series of flavonoids isolated from Indian medicinal plants were also analyzed for their action on XO. Nepetin and scutellarein were found to be the strongest inhibitors (9). It was known that the leaves of *Alsophila spinulosa* have been used as folk medicine for gout, hepatitis, rheumatism, and tumors. Caffeic acid was again the most potent constituent and demonstrated to be an uncompetitive XO inhibitor (10). Lastly, 34 crude extracts from Panamanian plants of the Celastraceae and Lamiaceae families were assayed for XO inhibitory activity. Eighty five percent of these extracts possessed XO inhibitory activity at 50 μg/ml (11). From this literature review, it can be summarized that flavonoids in general and several other phenolic derivatives appear to be the plant metabolites responsible for the XO inhibition. In the present paper we report the results of XO inhibition of 19 plant extracts of the Myrtaceae family collected in Puerto Rico.

Another condition investigated was cataractogenesis in streptozotocin (Sz) induced diabetic rats. The animals developed cataractogenesis after being hyperglycemic for three months. One of the possible causes for the acquisition of this condition is an increased lens hydration due to the accumulation of polyols in the lens. This buildup is produced by an increase in aldose reductase activity (12). However, other possible causes for developing cataracts are UV light , and exposure to oxyradicals. The oxyradicals include a very reactive species •OH derived from $H_2O_2$ (13). The normal concentration of $H_2O_2$ in the lens is 1-5 μM (14). This concentration is kept normally low by the effect of catalases and peroxidases but may increase under oxidation stress, as for example, in senile cataracts. It is known that $H_2O_2$ can lead to severe effects on critical cell functions because it interacts with DNA, cytosqueletal proteins and susceptible enzymes such as glyceraldehyde-3-phosphatedehydrogenase (G3PD). The $H_2O_2$ induced damage influence the cation transport systems in the lens (15).

It has been found that glutathione peroxidase and catalase also have a role in preventing cataractogenesis. Their protection to the lens is related to the lowering effect on $H_2O_2$ concentration. NADPH is needed to reduce glutathione which in turn reacts with $H_2O_2$ (16). Cataracts then, may be prevented by the action of reducing agents. NADPH is a reducing agent required by glutathione reductase for reducing oxidants and may have an application in the preclusion of lens opacity . Furthermore, NADPH prevents membrane lipid oxidation and permeability changes responsible for diabetic complications such as cataractogenesis and retinopathy (17).

A revision of the literature up to April, 1998 shows only one report of cataractogenesis inhibition by a plant extract. The fruit extract of *Momordica charantia* Linn. was studied in rats to evaluate the influence of this extract on the development of diabetic cataracts (18). The investigation of plant extracts that prevent cataractogenesis is important because it may lead to the discovery of agents which could be beneficial to diabetic patients. In this study we report the effects of two plant extracts one of which demonstrated activity on cataractogenesis inhibition.

## Materials and Methods

**Plant material.** Nineteen plant samples (leaves and stems) were collected in different places of the San Juan metropolitan area and Puerto Rico in May, 1993. The plants were identified by Mr. Carlos Rivera, a taxonomist of the Forest Service, Department of Agriculture of the United States. Voucher specimens have been deposited in the Herbarium of the School of Pharmacy, University of Puerto Rico. The leaves and stems were air dried, ground, and extracted with 95 percent ethanol. The resultant extracts were then filtered and concentrated by solvent evaporation.

**Assay of inhibitory activity**. The enzyme (XO), xanthine and allopurinol were purchased from Sigma Chemical Co., Saint Louis, Missouri. Allopurinol was used as a standard inhibitor. The XO activity was measured spectrophotometrically as follows:

Solutions of plant extracts were prepared by dissolving 2 mg of the extract in 1 ml of a 5% DMSO in water solution. This was added to 2.9 ml of 1.15 M phosphate buffer (pH = 7.5) and 0.1 ml of enzyme solution. After preincubation of the mixture at 25° for 15 min. the reaction was started by adding 2 ml of substrate solution and was stopped after 30 min. by adding 1 ml of 1N HCl. The absorbance was measured at 290 nm. A blank was prepared in the same way, with the enzyme being added to the assay mixture after the HCl. XO inhibitory activity was expressed as the percentage inhibition of XO and calculated by (1 - B/A) x 100, where A is the activity of the enzyme without the plant extract and B is the activity of the enzyme with the extract (4).

**Assay of extracts on cataractogenesis.** As of our results, *Syzygium malaccense* and *Eucalyptus deglupta* had the highest inhibitory activity. Aqueous extracts of each plant (0.06g/ ml) were assessed for possible prevention of cataractogenesis in Sz induced diabetic Sprague Dawley rats weighing 295-373 g. Groups of 5 rats were given the plant extract to drink *ad libitum* for 3 months while the controls received water. Two of the control and test animals died during the investigation due to unanticipated conditions. The groups were designated as A: rats with *E. deglupta*; B: rats with *S. malaccense*; C: diabetic rats; and D: non-diabetic rats .

## Results and Discussion

**XO inhibition.** Nineteen collections of leaves and stems of 19 species of the Myrtaceae family were extracted with ethanol (95%) and three sample replications were assayed for XO inhibitory activity at ±50 µg crude extract/ ml. Of the 19 assayed species only *Melaleuca quinquinervia, Pimenta racemosa, Psidium guajava* and *Syzygium jambos* (19,20) have a history of some medicinal use in Puerto Rican folk medicine. Only the data of leaves and young stems are reported here since these are the parts most usually employed in folk medicine The XO inhibitory activity results are listed in Table I.

All extracts showing enzyme inhibition ≥50%, were further tested to establish an $IC_{50}$ (µg/ml). The $IC_{50}$ and 95% confidence limits were calculated using the probit analysis method described by Finney (21). The inhibition of xanthine oxidase was demonstrated to be concentration dependent in all cases. Allopurinol consistently showed a high percentage inhibition. The inhibitory effect of the most active crude extracts compared to allopurinol is represented in figure 1.

*Syzygium malaccense* is an introduced species endemic to Malay peninsula or archipelago as its name suggests. This plant does not have reputed medicinal properties and it is not used in folk medicine in Puerto Rico. A Napralert literature search (22) shows no investigative reports in chemistry and pharmacology up to 1998. As for *Eucalyptus deglupta*, it is one of several closely related species of the genus *Eucalyptus* that grows in Puerto Rico. The species *E. globulus* and *E. maculata* are used as folk

**Table 1.** The inhibitory effects on XO by plant extracts of the Myrtaceae family.

| Scientific name | Common name | Percent o inhibition |
|---|---|---|
| 1. *Callistemon citrinis* | Cepillo | 40 |
| 2. *Eucalyptus crebra* | Eucalipto | 0 |
| 3. *Eucalyptus deglupta* | Eucalipto | 51 |
| 4. *Eucalyptus robusta* | Eucalipto | 29 |
| 5. *Eucalyptus peilita* | Eucalipto | 9 |
| 6. *Eucalyptus saligna* | Eucalipto | 0 |
| 7. *Eucalyptus tetericornis* | Eucalipto | 16 |
| 8. *Eugenia borinquensis** | Guayabota de sierra | 24 |
| 9. *Eugenia domingensis* | Guasábara | 0 |
| 10. *Eugenia stahlii** | Guayabota | 22 |
| 11. *Melaleuca quinquinervia* | Cayeputi, cajeput-tree | 13 |
| 12. *Myrcia deflexa* | Cieneguillo | 3 |
| 13. *Myrcia fallax* | Hoja menuda | 29 |
| 14. *Myrcia leptoclada* | Guayabacón | 13 |
| 15. *Myrcia splendens* | Hoja menuda, rama menuda | 0 |
| 16. *Pimenta racemosa* | Malagueta | 22 |
| 17. *Psidium guajava* | Guava, guayaba | 41 |
| 18. *Syzygium jambos* | Pomarrosa | 33 |
| 19. *Syzygium malaccense* | Manzana malaya | 64 |

* Endemic plant of Puerto Rico

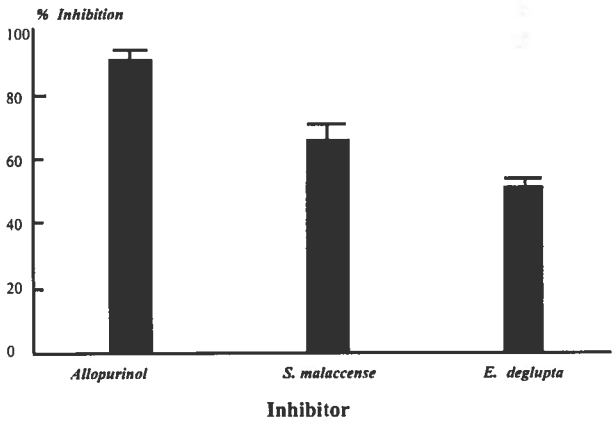


**Figure 1.** XO Inhibitory effect of *E. deglupta* and *S. malaccense* vs. Allopurinol. Percent of inhibition reflects the average of three experiments.

medicine in Puerto Rico (20). Since flavonoids have been reported as XO inhibitors (5), a phytochemical screening for these types of constituents was carried out with all extracts using the Shinoda test, obtaining positive results in all cases. The brine shrimp lethality test was also conducted with *S. malaccense* and *E. deglupta* producing $LC_{50}$ results about 1,000 µg/ml, suggesting the absence of cytotoxic, antifungal and insecticidal components (23).

**Effect on cataractogenesis.** Representative animals of each group are presented in Figures 2 to 5 after 3 months of ingestion of the plant extracts as previously described. Figure 2 shows a Sz induced diabetic rat that consumed *E. deglupta* extract. This group did not develop cataracts. The animals had an average glucose blood level of 411 mg/dl at the beginning of the experiment. Figure 3 presents a Sz induced diabetic rat that ingested *S. malaccense* extract. Rats in this group developed cataracts. The animals had an average glucose blood level of 341 mg/dl at the beginning of the experiment. Figure 4 represents an untreated Sz induced diabetic rat. Cataracts

**Figure 2.** An example of a group of 5 Streptozotocin (Sz) inducted diabetic rats that were allowed to drink *ad libitum* aqueous extract (0.06g/ml) of *E. deglupta* for three months. The animals had an average glucose blood level of 411 mg/dl at the beginning of the experiment.

**Figure 3.** An example of a group of 5 Streptozotocin (Sz) inducted diabetic rats that were allowed to drink *ad libitum* aqueous extract (0.06 g/ml) of *S. malaccense* for three months. The animals had an average glucose blood level of 341 mg/dl at the beginning of the experiment.

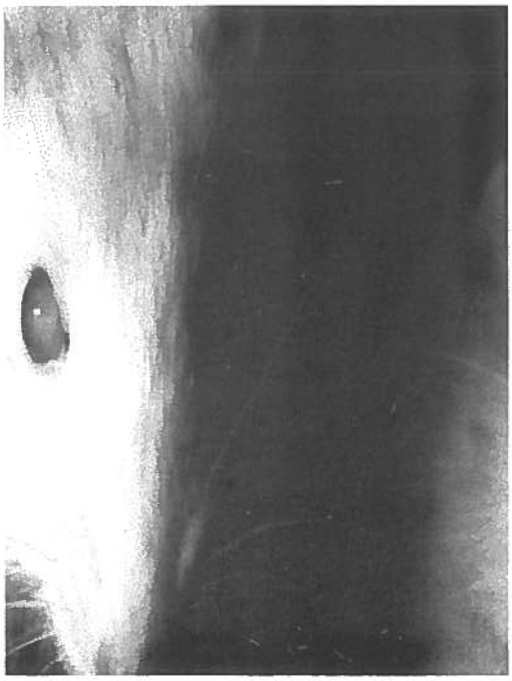

**Figure 4.** An example of a group of 5 untreated streptozotocin (Sz) induced diabetic rats that had an average glucose blood level of 373 mg/dl at the beginning of the experiment. The animals drank water *ad libitum*.

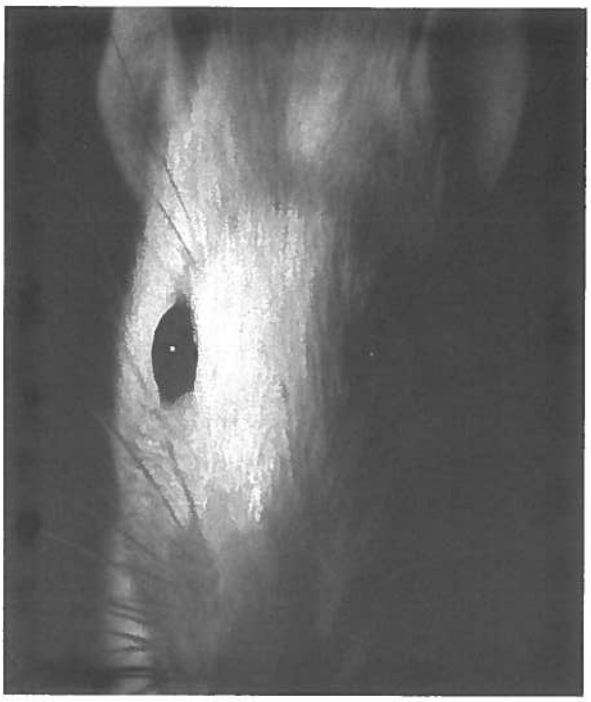

**Figure 5.** An example of a normal non diabetic rat from a group of 5. Their average glucose blood level was 82 mg/dl at the beginning of the experiment. The animals had drank water *ad libitum* during the three months of the experiment.

were observed in all animals of this group. The animals had an average glucose blood level of 373 mg/dl at the beginning of the experiment. Figure 5 presents a rat of the non-diabetic group which did not develop cataracts. Their average glucose blood levels were 82 mg/dl at the beginning of the experiment.

In our study of the inhibition of cataractogenesis, plant extracts were provided to test and control animals at a concentration of 0.06 g/ml during a 3 month period. This concentration is much higher than the *in vitro* $IC_{50}$ obtained for the extracts (51 µg/ml for *S. malaccense* and 45 µg/ml for *E. deglupta*). The protection of *E. deglupta* against cataractogenesis in Sz induced diabetic rats is due to the active constituents of the extract. This extract was found to contain flavonoids according to Shinoda reaction. Flavonoids are known to have XO inhibitory activity in the body tissues and may be the responsible metabolites for this action. Another argument could be that the extract may have a protective effect through an increased aldose reductase inhibitory activity. As for *S. malaccense*, the extract was unable to protect the rats against cataractogenesis. At present, the XO inhibitors of *E. deglupta* and *S. malaccense* have not been identified, much less the concentration at which they are present in these two extracts. However, a high flavonoid concentration or the presence of a different active metabolite in *E. deglupta* may be responsible for the inhibition of XO and the observed anti cataractogenic activity.

*E. deglupta* extract is currently under investigation with two objectives in mind. First, to achieve the isolation and structural identification of the active metabolite(s), and second, to determine if the active chemical species are antioxidants that work as scavengers of superoxides generated by the hypoxanthine/xanthine oxidase.

## Resumen

El objetivo de este trabajo consistió en investigar nuevos inhibidores de xantina oxidasa y analizar su posible efecto en la prevención de cataratogénesis. Para este fin se seleccionaron diecinueve especies de plantas de la familia Mirtácea en Puerto Rico. Las hojas y tallos de estas plantas se secaron, molieron y maceraron con alcohol etílico al 95%. Después de la evaporación del solvente, los extractos fueron examinados espectrofotométricamente por la inhibición de xantina oxidasa. Se descubrió que los extractos de *Syzygium malaccense*($IC_{50}$ 51µg/ml) y *Eucalyptus deglupta* ($IC_{50}$ 44.5 µg/ml) mostraron la mejor inhibición. Además, los extractos acuosos de estas dos plantas (0.06 g/ml) se estudiaron por una posible prevención de cataratogénesis usando ratas diabéticas (Sprague Dawley), en donde la diabetes fue inducida por estreptozotocina. Grupos de dos o tres ratas diabéticas ingerieron extractos de plantas *ad libitum* por tres meses mientras que los controles sólo bebían agua. Al final del experimento, solamente el grupo de *E. deglupta* y el control no-diabético no habían desarrollado cataratas.

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