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EFFECT OF SOFT DRINKS ON *IN-VITRO* KIDNEY STONE (CALCIUM OXALATE MONOHYDRATE)

Ayesha Tajammul [1], Nurul Kabir [1], Maryam Ahmed [*2] and Ali Sufyan [3]

Dr. Panjwani Center for Molecular Medicine and Drug Research [1], International Center for Chemical and Biological Sciences (ICCBS), University of Karachi, Karachi, Pakistan.
Department of Pharmacognosy, Faculty of Pharmacy [2], Federal Urdu University of Arts, Science and Technology, Karachi, Pakistan.
Department of Microbiology [3], University of Karachi, Karachi, Pakistan.

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Correspondence to Author:
Maryam Ahmed

Assistant Professor,
Faculty of Pharmacy,
Federal Urdu University of Arts,
Science and Technology, Karachi,
Pakistan.

E-mail: maryamahd@yahoo.com

ABSTRACT: Kidney stone is a painful disease which is a major problem in all regions of Pakistan as this country is located in the so-called stone belt. Drastic changes in dietary habits including the use of soft drinks are increasing the incidence of kidney stone disease. To investigate this phenomenon, calcium oxalate monohydrate crystals were induced in artificial urine by mixing calcium chloride and sodium oxalate *in-vitro* and different types of soft drinks were analyzed for activity against calcium oxalate monohydrate type's kidney stone. Most of them showed effects on calcium oxalate monohydrate to calcium oxalate dihydrate crystals under DIC microscopy. This study showed that slices had the most promising effect on calcium oxalate monohydrate crystals than Mirinda and Mountain dew. 7up and Pepsi did not show a change in morphology of the crystals of calcium oxalate monohydrate.

INTRODUCTION: Kidney stone disease is as old as humans and has been found in the tombs of Egyptian mummies dating back to 4000 BC [1]. In the past, it was considered a disease with terrible upshots which frequently lead to death. The epidemiology of kidney stone in a given population varies according to the geographical area, climate and season. Pakistan is situated in the Afro-Asian stone belt [2]. Most of the cases of kidney stones have been reported in the summer rather than the winter in a given population [3]. People of all ages are susceptible to kidney stone disease.

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Prevalence of kidney stone varies according to the sex, and 20% of the females and 4% of the males are affected by this disease [4]. The changes in dietary habits, such as having fast food rather than traditional food, increase the chance of stone formation, as well as abnormal intake of fast foods, soft drinks, sodium, and oxalate-containing vegetables and fruits take part in the progression of kidney stone disease [5].

Calcium oxalate crystallization is a very complex and fundamental step in the formation of kidney stones. This process includes; urinary super-saturation, crystal nucleation, crystal growth, aggregation, and crystal-cell interaction. Many researchers have proposed *in-vitro* assay to evaluate the activity of different plant's extracts along with their isolated or synthesized compounds against kidney stone [6]. This study is based on *in-vitro* kidney stones formation in artificial urine

containing pathological calcium oxalate monohydrate crystals and their modulation or morphological change into non-pathological calcium oxalate dihydrate crystals [7] by a variety of soft drinks acting against these kidney stones *in-vitro*. This activity can be determined by spectrophotometry [8] and microscopy [9].

MATERIALS AND METHODS:

Soft Drinks: Different varieties of soft drinks were purchased from the departmental store of Karachi which included Slice, Mirinda, Mountain dew, 7up, and Pepsi. Approximately 5, 10, 15 and 20 µL of each drink were used for this study to evaluate the activity against calcium oxalate monohydrate kidney stone.

Spectrophotometric Analysis: After making artificial urine in a 96-well flat-bottomed plate by mixing 100 µL calcium chloride (4 mmol/L), 100 µL sodium oxalate (0.5 mmol/L), 200 mmol/L sodium chloride and 10 mmol/L sodium acetate, calcium oxalate stones were induced *in-vitro* [8]. This artificial urine was adjusted to 5.7 pH. The optical density was measured after each five minutes interval throughout 80 min at 620 nm at 37 °C using a Sunrise Microplate Reader (Tecan, Austria GmbH) with and without the addition of different soft drinks during crystallization in artificial urine.

Microscopic Studies: After the spectrophotometric determination, the effects of soft drinks were observed on calcium oxalate monohydrate crystals under the Differential Interference Contrast (DIC) microscope. Using polarized light, crystals were distinguished on the bases of their morphology. A Nikon Eclipse TE2000-E microscope (Nikon, Japan) and Nikon DXM 1200C camera were used to obtaining the images. The images were analyzed by using NIS-Elements image analysis software AR 3.2 (Nikon, Japan).

Statistics: Statistical calculations were performed by Microsoft Excel 2003 and p-value of 0.001-0.05 was measured to be significant in t-tests.

RESULTS AND DISCUSSION: To determine the activity of soft drinks against kidney stone *in-vitro*, calcium oxalate monohydrate crystallization were analyzed by spectrophotometry and observed under DIC microscope and results are described in **Table 1**.

TABLE 1: SPECTROPHOTOMETERIC AND MICROSCOPIC DETERMINATION OF EFFECT OF SOFT DRINKS AGAINST CALCIUM OXALATE CRYSTALLIZATION

S. no.	Name of soft drink	5 µl/mL	10 µl/mL	15 µl/mL	20 µl/mL
1	Slice	+	++	+++	++++
2	Mirinda	+	+	+	+
3	Pepsi	-	-	-	-
4	Mountain Dew	+	+	+	+
5	7up	-	-	-	+

Spectrophotometric Analysis: Slice showed excellent while mirinda and mountain dew showed moderate activity against calcium oxalate monohydrate crystals *in-vitro*. Pepsi and 7up did not show noticeable activity against calcium oxalate monohydrate kidney stone.

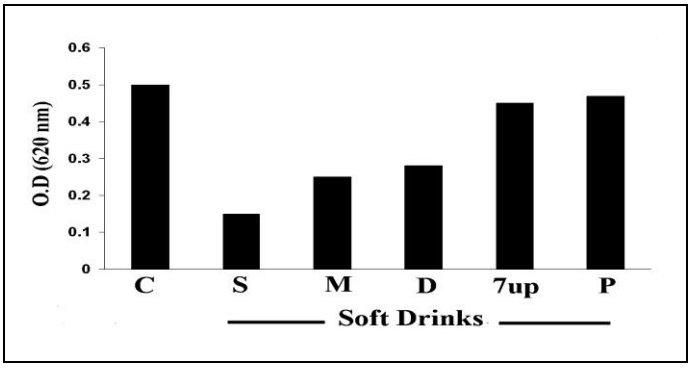

FIG. 1: SPECTROPHOTOMETRIC ANALYSIS OF SOFT DRINKS AGAINST CALCIUM OXALATE CRYSTALLIZATION IN ARTIFICIAL URINE. *In-vitro*, calcium oxalate monohydrate stone was induced and optical density was measured at 620 nm throughout 80 min. Slice decreased the optical density more significantly than other soft drinks. C, S, M, D, and P represent the control group, Slice, Mirinda, dew and Pepsi respectively.

Spectrophotometric analysis **Fig. 1** showed that slice has considerable effect against *in-vitro* calcium oxalate monohydrate crystals than Mirinda and Mountain dew by decreasing the optical density at 620 nm as compared with control. No change was observed in the optical density of 7up and Pepsi when reading against a control group containing calcium oxalate monohydrate crystals.

Microscopic Determination:

Slice: Elongated rod-shaped calcium oxalate monohydrate (COM) crystals were observed under DIC microscope **Fig. 2A** in 200x magnification with 15 different fields. Slice juice with a dosage of 5, 10, 15 and 20 µL/mL were used to evaluate the activity against COM *in-vitro* kidney stones. Pyramidal shaped calcium oxalate dihydrate (COD) stone was observed under DIC microscope when treated with a slice **Fig. 2B**. Approximately 16 ± 3.3 COM crystals were observed under DIC microscope **Fig. 3A** and their size was 150 ± 1 μm^2 under control conditions **Fig. 4A**. The numbers of the stones **Fig. 3A** were found 18 ± 1.1, 20 ± 4.2, 28 ± 7.5 and 34 ± 6.0 with 5, 10, 15 and 20 µL/mL of slice juice respectively. The sizes of the stones **Fig. 4A** were 286.1 ± 1.3, 252 ± 3.6, 199.3 ± 4.8 and 172 ± 9.1 μm^2 with 5, 10, 15 and 20 µL/mL of slice respectively. Slice showed its effect as dose-dependent increased in stone number and decrease in stone size which suggested significant activity against *in-vitro* COM kidney stone.

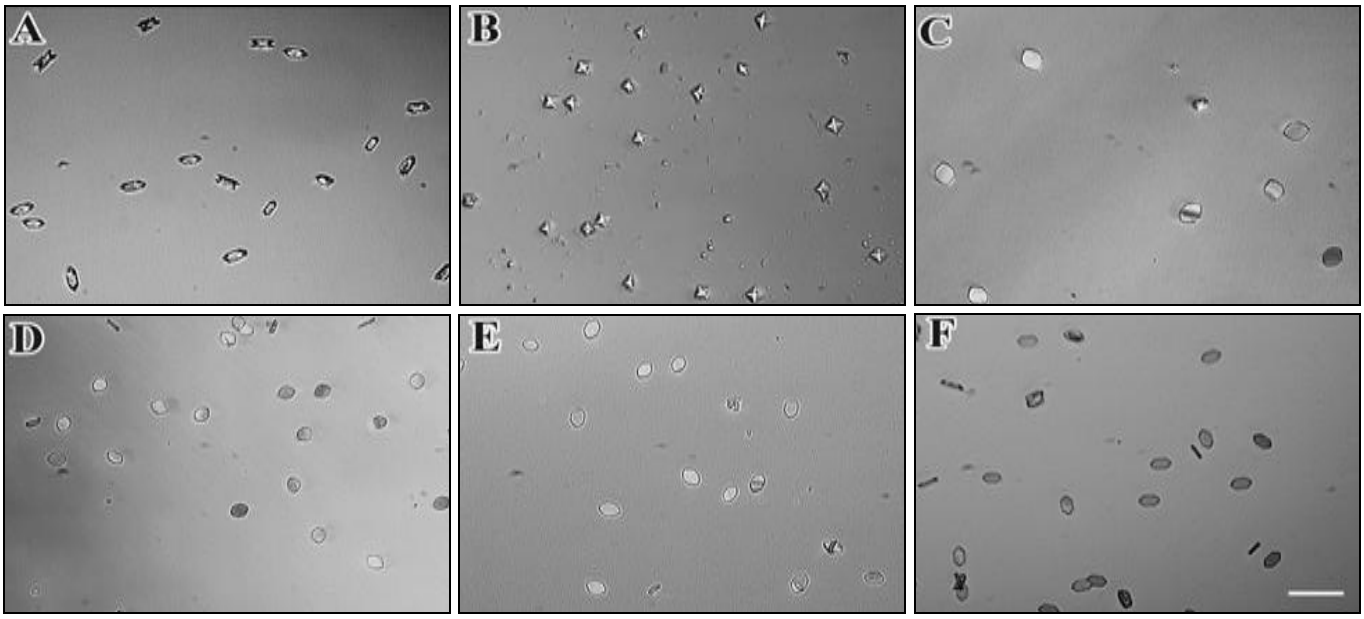

FIG. 2: *IN-VITRO* EFFECT OF SOFT DRINKS ON KIDNEY STONE CRYSTAL'S MORPHOLOGY WAS OBSERVED BY DIC MICROSCOPY. *In-vitro* assay against kidney stone was observed under DIC microscopy at a magnification of 200X. Under control conditions (A) only rod-shaped calcium oxalate monohydrate crystals were observed. At constant dose (15 µl/mL) of different soft drinks, pyramidal-shaped COD crystals were observed with a slice (B) whereas hexagonal shaped crystals were observed with mirinda (C) and mountain dew (D). No or very little effects were observed with pepsi (E) and 7up (F) respectively. Scale bar is 50 µm

Mirinda and Mountain Dew: Spectrophotometric assay suggested the moderate activity of the mirinda and mountain dew against the formation of the kidney stone **Fig. 1** as compared with control. When COM crystals were treated with mirinda and mountain dew hexagonal shaped calcium oxalate crystals were observed in the similar dosage of 5, 10, 15 and 20µL/mL of mirinda **Fig. 2C** and mountain dew **Fig. 2D** under DIC microscope. These types of crystals are also reported as in inert form to bind with damaged kidney cells [10].

The number of the crystals in 200x magnification field were 16 ± 3.3, 15 ± 1.0, 19 ± 11.6, 17 ± 13.1, 18 ± 7.3 with control, 5, 10, 15 and 20 µL/mL of mirinda **Fig. 3B** respectively. Whereas, the number of the crystals were 16 ± 3.3, 5 ± 7.1, 8 ± 15.1, 13 ± 5.1, 19 ± 7.3 with control, 5, 10, 15 and 20 µL/mL of mountain dew **Fig. 3C** respectively. The sizes of the stones were 150 ± 1, 299.9 ± 17.0, 352.1 ± 11.0, 328.9 ± 14.8, and 179.9 ± 10.7 μm^2 with control, 5, 10, 15 and 20 µL/mL of mirinda **Fig. 4B** respectively.

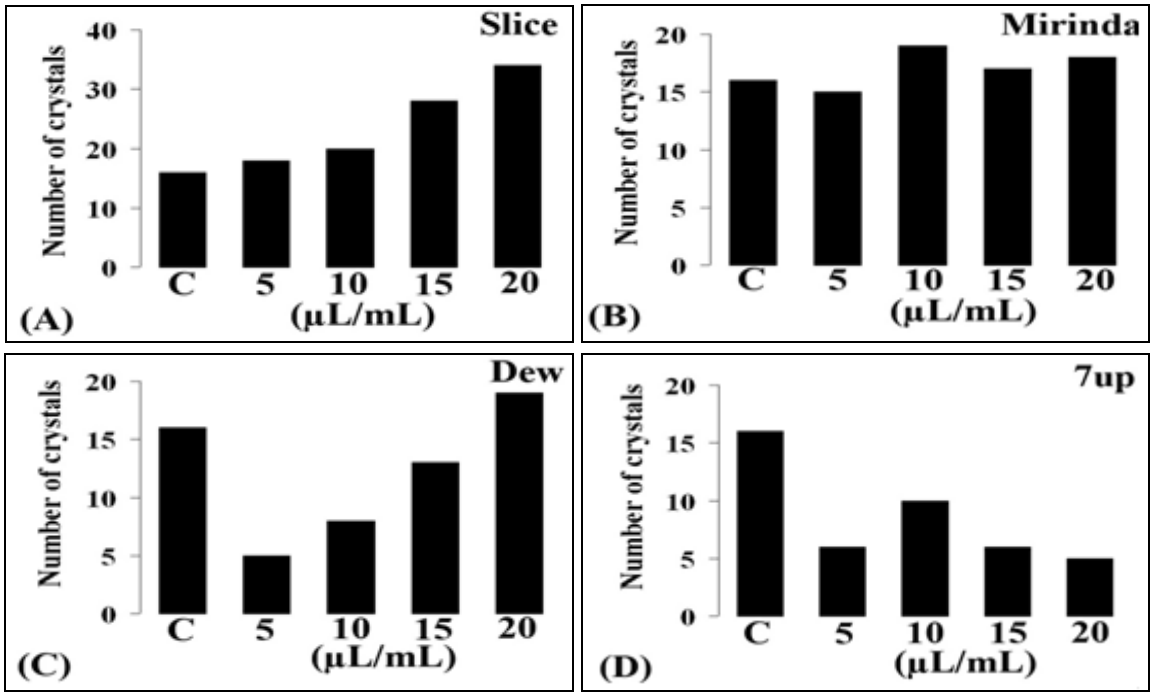

FIG. 3: *IN-VITRO* EFFECT OF DIFFERENT DOSAGE OF SOFT DRINKS ON COUNTING OF CALCIUM OXALATE STONES. Soft drinks affected on a number of stone by different manners and showed activity against calcium oxalate crystals. Slice (A), Mirinda (B), Mew (C) and 7up (D) showed different effects on crystals number.

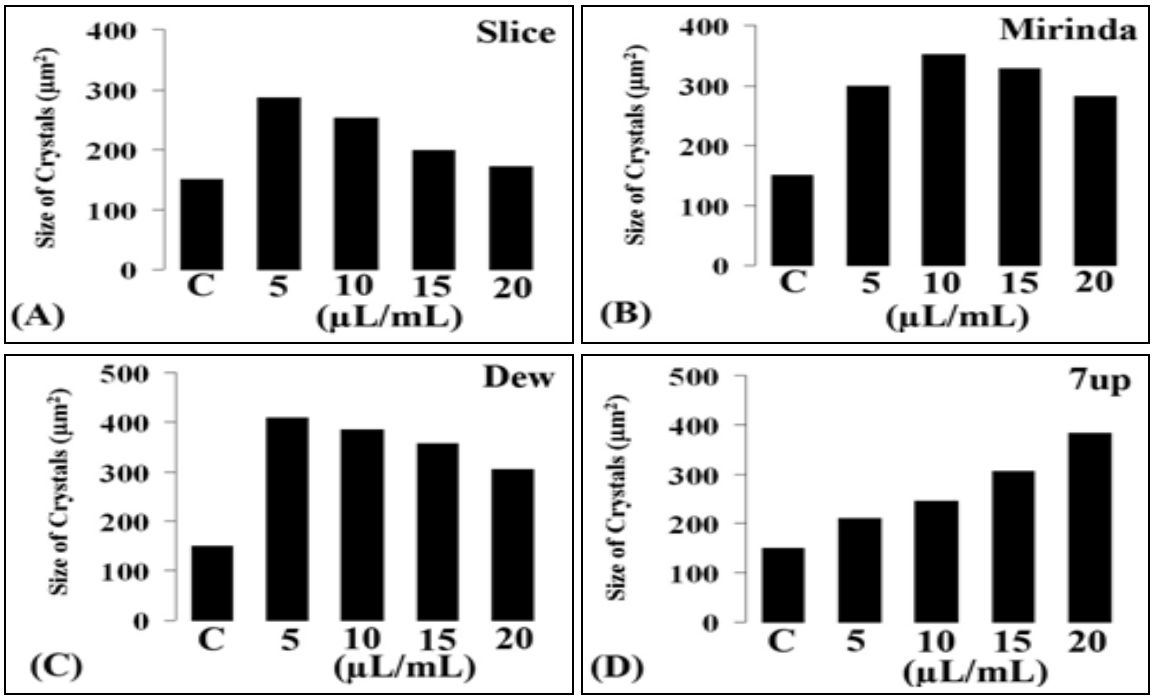

FIG. 4: *IN-VITRO* EFFECT OF DIFFERENT DOSAGE OF SOFT DRINKS ON SIZE OF CALCIUM OXALATE STONES. Soft drinks affected on the size of stone by different manners and showed activity against calcium oxalate monohydrate crystals. Slice (A), Mirinda (B), Dew (C) and 7up (D) showed different effects on crystal size.

Whereas the sizes of the stones were 150 ± 1, 409 ± 10.0, 385 ± 9.0, 356.7 ± 8.2 and 304 ± 13.3 µm^2 with control, 5, 10, 15 and 20 µL/mL of mountain dew **Fig. 4C** respectively. These changes in morphology of COM crystals may be due to the chelation of calcium by citrate present in both Mirinda and Mountain dew. These changes were not similar as observed in the slice, therefore, showed moderate activity against *in-vitro* calcium oxalate kidney stones.

7up and Pepsi: 7up & pepsi did not show activity against *in-vitro* calcium oxalate monohydrate kidney stones. Under DIC microscopic examination

with 200x magnification, the shape of stones was observed with the rod to hexagonal with increasing in size and decreasing in a number of the crystals after treatment with 7up **Fig. 2E**, but the effect was opposite to Mirinda and Mountain dew with similar dosage. The numbers of the stones **Fig. 3D** were found 16 ± 3.3, 16 ± 5.1, 10 ± 14.2, 6 ± 17.5 and 5 ± 8.0 with control, 5, 10, 15 and 20 µL/mL of 7up respectively. The sizes of the stones **Fig. 4D** were 150 ± 1, 210.1 ± 5.3, 245 ± 12, 305.2 ± 12.1 and 384 ± 11.1 μm^2 with control, 5, 10, 15 and 20 µL/mL of 7up respectively. DIC microscopic examination confirmed the presence of COM crystals even after treatment of control crystals to Pepsi **Fig. 2F** which did not show any conformational change in crystals morphology [11-13].

CONCLUSION: In this study, we suggested that soft drinks effect on calcium oxalate monohydrate kidney stone. Effect of soft drinks including Slice, Mirinda, Mountain dew, 7up and Pepsi was evaluated to determine *in-vitro* activity against calcium oxalate kidney stones by using spectro-photometry and DIC microscope. Slice was observed with significant and Mirinda as well as mountain dew showed moderate activity against kidney stone whereas 7up and Pepsi did not show any effect against *in-vitro* calcium oxalate monohydrate crystals. Slice, Mirinda and Mountain dew greatly affected the stone formation and reduced the stone size but increased the stone number.

These changes in crystals morphology are beneficial because COM crystals are associated to bind with cells of the damaged kidney to produce kidney stone disease. Physiological COD crystals are harmless because they are less able to adherence to damaged kidney cells and excreted out from the body through the urination.

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CONFLICT OF INTEREST: Nil

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