

Detection of Rutin, Kaepferol, and Quercetin based Crude from Corn Silk and Studying their Effects on the Inhibition of Pure Urease Enzyme and Urease of *Klebsiella* Species

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ABSTRACT

This paper was carried out for detection of Rutin, Kaepferol, and Quercetin in corn silk, and an investigation of their effects (within crude) on the inhibition of urease enzyme and *Klebsiella* species urease. An HPLC has been used to detect the three flavonoid components in three extracts of corn silk (using 99.9% ethanol, 80% ethanol and water). The results were (0.012mg/L) of Quercetin in 99.9% ethanolic extract (99.9% EE). (0.1398 mg/L), (0.15 mg/L), and (0.11 mg/L) of Rutin, Kaepferol, and Quercetin respectively in 80% ethanolic extract (80% EE). (0.071 mg/L) and (0.091 mg/L) of Rutin and Kaepferol respectively in aqueous extract (AE). It is noticed that only 80% ethanol has an ability to extract such three flavonoids. All such extracts revealed an effective inhibition of urease enzyme in the *Klebsiella* species with (IC₅₀ = 77.06, 13.54 +- and 35.93 mg/L) for 99.9% ethanol, 80% ethanol and aqueous extracts respectively, while standard urease inhibitor exhibits (IC₅₀ = 453.4 mg/L) for thiourea (TU) as an example. Lastly, such extracts were utilized in the inhibition of pure enzyme and they exhibited an efficient inhibition of (IC₅₀ = 402.8, 95.8, and 348 mg/L) for 99.9% ethanol, 80% ethanol and aqueous extracts respectively as compared with standard urease inhibitor of which gives (IC₅₀ = 54077 mg/L) in thiourea (UT).

Keywords

Ethanol, Flavonoid,
Urease inhibition,
HPLC, Rutin,
Quercetin, Kaepferol.

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Introduction

Corn silk

Corn silk is a long, weak, and shiny fibers at the top of corn's ear (1). Traditionally, it is used for making tea as a healthy and medical drink in Asian communities especially in China (2). Recently, corn silk becomes very important in drugs development, because of its bioactive constituents which include oxidant prevention agent limits, anti-diabetic activity anti-proliferative effects diuretic

activity anticoagulant activity, antifungal, anti-fatigue, and treating obesity (3).

In addition, corn silk has been used as a treatment of many diseases like hyperglycemia, hypertension, cystitis, tumor, hepatitis, gout, diabetes, kidney stones, prostatitis, and nephritis (4). Moreover, it is used medicinally as a mellow stimulant, diuretic, and demulcent. It is helpful in intense and incessant cystitis and in the

bladder aggravation of uric corrosive, phosphatic grave, and employed in Gonorrhoea (5). According to the phytochemical studies on corn silk, it revealed that corn silk contains a number of components like protein, vitamin, some minerals (Ca, K, Mg, Mn, and Zn), flavonoids, steroid, carbohydrate and volatile components (4, 6). Besides that, it contains chlorogenic acid, p-coumaric, ferulic acid, saponins, phytosterols, volatile oil, fixed oil, resin, sugars, allantoin, and tannin (5).

Flavonoids

Physiologically, Flavonoids (the original term is a Latin name called "flavus" word; which means yellow) play a vital role as the major former of blue, red, and purple pigments of plants tissues (7). Phenol was considered as a dominant component in flavonoid formation, the three carbon atoms were formatted for connecting two rings of phenol to form a heterocyclic ring by joining them with an oxygen atom (8). Flavonoids are the essential group of polyphenolic. They exhibit important effects on radicals' scavengers and health strengthen properties. Polyphenols have abilities to inhibit free radicals, to decrease the cardiovascular problems, and to produce a strong anti-inflammatory and anti-cancer activity (9). Flavonoids effectively used to treat the obesity, hypertension, and dyslipidemia in both humans and animals (10). Flavonoids fall into several types like flavones, flavanones, anthocyanins isoflavones, and flavonols (11).

Quercetin

It is a plant polyphenol belongs to flavonol of flavonoids. Mostly, It's available in many human foods especially fruits, leaves, grains, and vegetables (12). It characterizes by a phenyl benzo (c) pyrone derived structure as represented by its chemical structure shown in figure 1 (13).

Rutin

It is the glycoside which combine the disaccharide rutinose (α -L-rhamnopyranosyl-(1 \rightarrow 6)- β -D-glucopyranose) and the flavonol quercetin (10). Lots of researches proved the significant effects on preventing several types of diseases (14). It has several pharmacological characteristics like anti-inflammatory, antioxidant, anti-carcinogenic, antiviral, antiallergic, and strong scavenger of superoxide radicals (15). The chemical structure of rutin is exhibited in figure 2.

Kaepferol

It is known as a natural flavonol (one of the flavonoids type). It is one of the most common food flavonoids; initially, it found in tea propolis, and grape fruit (16). It is popular due to its antioxidant activity and his efficient utilization in cytoprotection agents. Previous experiments revealed that Kaepferol produces anti-proliferation activity and contains apoptosis in a variety of human cancer cell lines in vitro, for examples, leukemia, and cancers related to non-small cell lung, prostate, esophageal, cell lung, colon, oral cavity (15). Figure 3 presents the chemical structure of Kaepferol.

***Klebsiella* species**

It is a gram negative bacterium in the family of Enterobacteriaceae. It is heavily dispersed in water, air, and soil. It forms a part of the normal microbiota which exists in human and animal gastrointestinal and urinary tracts (17).

It produces urease enzyme which hydrolysis of urea and its last items being alkali and carbonic acid (18). Ureases are multi-subunit of nickel-containing enzymes (19). They are playing very important role in several pathological situations such as gastric cancers, peptic ulcers, hepatic

encephalopathy, urinary catheter encrustation, and urolithiasis. Its inhibition, therefore; has a major therapeutic (20). However, Ureases inhibited by numbers of compounds such as hydroxamic acids, phosphoramidites, and imidazoles. Several studies of inhibition have been carried out to investigate the molecular mechanism of urease activity moreover to assure compounds that could effectively control its activity (18).

Materials and Methods

Extraction from corn silk

The samples of corn plant were collected at harvesting time where their materials are fully matured and developed. Firstly, the corn silk flowers were gathered from corn fields of the faculty of Agriculture's farm of Baghdad University in February 2017. Secondly, they were dried in a shaded well-ventilated place. Thirdly, pulverizing them using a knife mills then keeping them stored in glass containers at room temperature for further processing (21). A 100 g of pulverized corn silk were exposed to a hot continuous extraction by Erlenmeyer flask in a Ultrasonic with 1L of (99% ethanol, 80% v/v ethanol/water and water) (3*5 L) at steady temperature of (50 ± 1.0 °C) for three cycles of 5 hours periods for each. Each of three previous extracts was filtered through Whatman No. 1 filter paper to remove the debris. Then, each filtered sample was condensed by a rotary flash evaporator under vacuum at 50°C. Finally lyophilizing each condensed samples in a freeze-dryer to obtain a crude ethanol Extract (EF), 80% ethanolic extract, and water extract. Lastly, all extracts were stored at 4 °C for subsequent analysis (12). The below equation used to determine the yield as percentage of the quantity of the initial material of (100g).

$$\text{Yield \%} = \frac{\text{yield} * 100}{100 \text{ g}}$$

Detection some flavonoids by HPLC

A 0.1g of each crude was dissolved in methanol of volume 100 mL. A 20 µL of each dissolved was filtered through 0.45 µm membrane then analyzing them for detecting the compounds (Rutin, Kaepferol, Quercetin, and Maysin) using reversed phase HPLC, column= (250*4.6mm Id) 5 mm particle size. Then diluting with methanol-water (80 to 100%) and methanol with 1% Orthophosphoric acid.

The UV-Vis detector set at 280 nm. A 0.1g of each crude was dissolved in methanol of volume 100 mL. A 20 µL of each dissolved was filtered through 0.45 µm membrane then analyzing them for detecting the compounds (Rutin, Kaepferol, Quercetin, and Maysin) using reversed phase HPLC, column= (250*4.6mm Id) 5 mm particle size. Then diluting with methanol-water (80 to 100%) and methanol with 1% Orthophosphoric acid. The UV-Vis detector set at 280 nm.

The place of flavonoids in the HPLC results was defined through comparing the peak retention time between extracts and standard flavonoids solution; (retention time of compound Rutin, Kaempferol, and Quercetin were 2.85, 3.08, and 7.31 min; respectively).

The content of flavonoids was calculated through regression equation between peak area and flavonoids content (8).

Preparation concentration

To prepare the stock solutions; initially, all extracts were dissolved with phosphate-Buffered (PH =7). Then, diluting the previous concentrations of (1000, 500, 250, 125, and 62.5) mg\L with phosphate-Buffered (PH =7). At the end, all extracts were filtered with a micro filter of 0.45 µm then storing them at 4 °C for further use.

Activation of microorganisms

The specimen of the colonies was taken by a loop that contains 5 ml of sterilized nutrient broth. The loop has been shaken well and incubated in the incubator for 24 hours at 37 °C. The loop was sterilized via flame before using it to ensure that the planted bacteria are not contaminated.

***Klebsiella* species urease inhibition assay**

Dissolve 38.71 g of urea broth powder in 1000 ml distilled water. Then, thoroughly mix to dissolve the medium completely then sterilize the results by Autoclave. After that, 40% urea was sterilized by filtration. After activation, under a sterile tube and aseptic ambience, the desired colony was taken by a loop to the test tube that contains 5 ml of the sterilized urea broth.

The inhibition of urease examination was performed spectrophotometrically in 96-well Microplate. The solution: (100µl) of bacteria diluted of *Klebsiella* species was incubated with 100µl of Extracts (99.9% EE), (80% EE), and (AE) in concentrations of (1000, 500, 250, 125, 62.5, and 31.25 mg/L) at 30 °C for 24 hour. Thereafter, Urease activity was continuously measured with the rate of ammonia generation, and change in absorbance (optical density) was measured at 630 nm on ELISA plate reader in Compare with standard urease inhibitor i.e. thiourea (TU) (20).

Urease inhibition assay in pure enzyme

The urease inhibition assay has been run spectrophotometrically in 96-well plate. A phosphate buffer (pH 6.8; 4 mM) has been used to dissolve solution of urease enzyme. The net reaction volume was 200 µL. A 25µ L of urease enzyme incubated with 5 µL of Extracts (99.9%EE), (80%EE), and (AE) in

concentration (1000, 500, 250, 125, 62.5, and 31.25 mg/L) at 30 °C for 15 minutes. After that, Urea (55 µL; 100 mM) was added and the plate was incubated again at 30 °C for 10 minutes. After incubation, the results added to each well 70 µL of alkali reagents (sodium hydroxide (0.5% w/v) and sodium hypochlorite (0.1%)) and 45µL of phenol (pheno1(% w/v) and sodium nitroprusside (0.005% w/v)); The plate was again incubated at 30 °C for 50 minutes. The activity of urease was continuously estimated with the rate of release of ammonia, and the absorbance changing (optical density) was observed at 360 nm on ELISA plate reader. Thiourea used as a standard compound (20).

Statistical analysis

All experiments were carried out in triplicate. The results were processed via Microsoft excel. The percentage of inhibition was calculated by using the formula given below.

$$\% \text{ Inhibition} = 100 - \left\{ \frac{\text{Absorbance of Test Compound}}{\text{Absorbance of Control}} \times 100 \right\}$$

Measuring the effects of different concentrations of inhibitors on production of ammonia was used to evaluate the IC₅₀ of the active compounds. The IC₅₀ values were determined using Graphpad Prism7 software.

Results and Discussion

Plant extract

The yields of corn silk's extracts with respect to the solvent are shown in table 1. The percentage of yields calculated according to 100g of corn silk for each extraction method.

According to the results shown in table 1, it is noticed that the water solvent gives higher yield than others which can considered as factor in solvent cost reduction.

Table.1 The extracted yields for every relevant solvent

Type of solvents used in extraction	Yield(g)	yield%
99.9%ETHANOL	0.93	0.93%
80%ETHANOL	2.4	2.40%
WATER	9.13	9.10%

Table.2 Concentrations of (Rutin, Kaepferol and Quercetin) in (99.9%EE) (80%EE), and (AE)

Type of flavonoids	R time (min.)	Concentration in 99.9% ETHANOL extract (mg/L)	Concentration in 80% ETHANOL extract (mg/L)	Concentration in aqueous extract (mg/L)
Rutin	2.851	-	0.1398	0.071
Kaepferol	3.08	-	0.15	0.091
Quercetin	7.315	0.012	0.11	-

Table.3 *Klebsiella* species urease inhibitory activity of Extracts (99.9%EE) (80%EE), (AE) as compared with standard urease inhibitor thiourea (TU)

conc. (mg/L)	Inhibition% of (TU)	Inhibition% of.(99.9%EE)	Inhibition% of(80%EE)	Inhibition% of (AE)
31.25	29.78	23.72	9.92	28.81
62.5	34.62	29.53	23.97	34.62
125	38.25	33.89	31.23	37.77
250	39.7	38.98	32.62	39.22
500	45.76	41.64	36.25	40.67
1000	52.3	44.33	42.42	44.3

Table.4 IC₅₀+SEM inhibitor in *Klebsiella* species urease

Type of inhibitor	IC ₅₀ +SEM(mg/L) (inhibitory in <i>Klebsiella</i> Species urease)
Thiourea (TU)	453.4 ± 1.84
99% ethanol extraction(99.9EE)	77.06 ± 1.19
80% ethanol extraction(80%EE)	13.54 ± 4.02
Aqueous Extraction (AE)	35.93±2.37

Table.5 Urease inhibitory activity of extracts (99.9%EE), (80%EE), and (AE) in comparative to standard urease inhibitor i.e. thiourea (TU)

Concentration (mg/L)	Inhibition % of (TU)	Inhibition % of.(99.9%EE)	Inhibition% of(80%EE)	Inhibition% of (AE)
31.25	37.65	33.77	33.4	33.61
62.5	39.64	35.08	36.1	36.18
125	42.31	38.07	39.9	37.65
250	45.2	39.17	41.26	39.95
500	46.51	43.36	42.42	44.57
1000	57.99	46.3	45.98	46.82

Table.6 IC50+SEM inhibitor in pure urease enzyme

Type of inhibitor	IC50+SEM(mg/L) (inhibitory in pure urease enzyme)
Thiourea (TU)	54077
99%ethanol extraction(99.9EE)	402.8
80%ethanol extraction(80%EE)	95.8
Aqueous extraction (AE)	348

Fig.1 Chemical structure of Quercetin

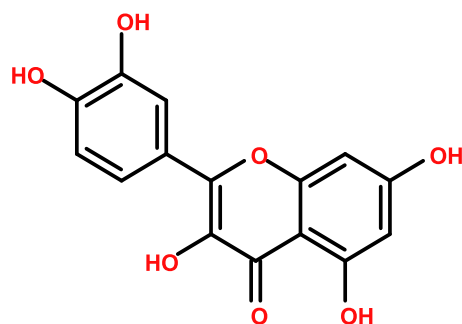


Fig.2 The chemical structure of Rutin

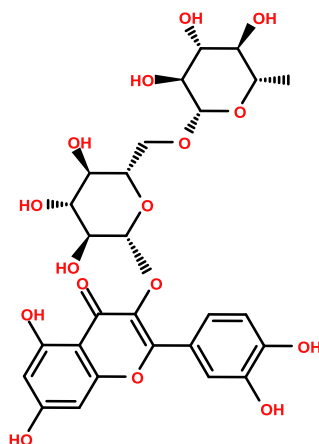


Fig.3 Chemical structure Kaepferol

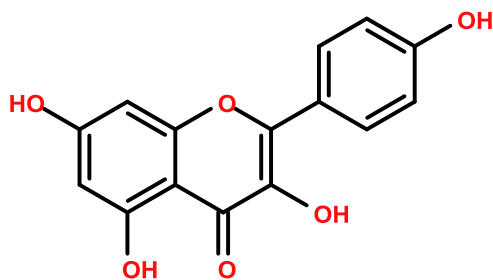


Fig.4 HPLC of standard flavonoids (retention time of Rutin, Kaepferol, and Quarcetin, were 2.85, 3.08, and 7.31 min, respectively)

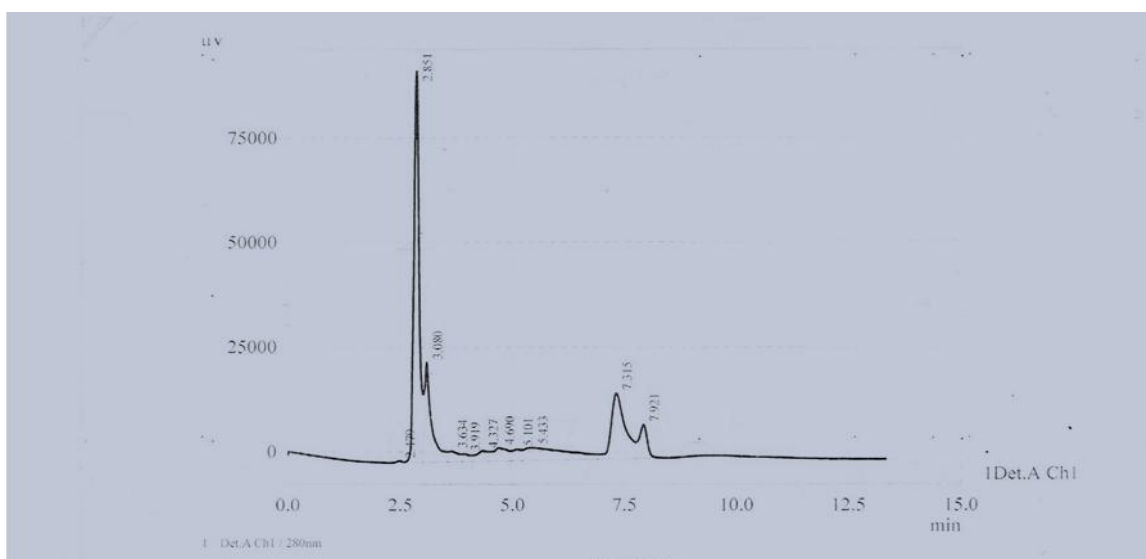


Fig.5 HPLC of 99.9% ethanolic extract

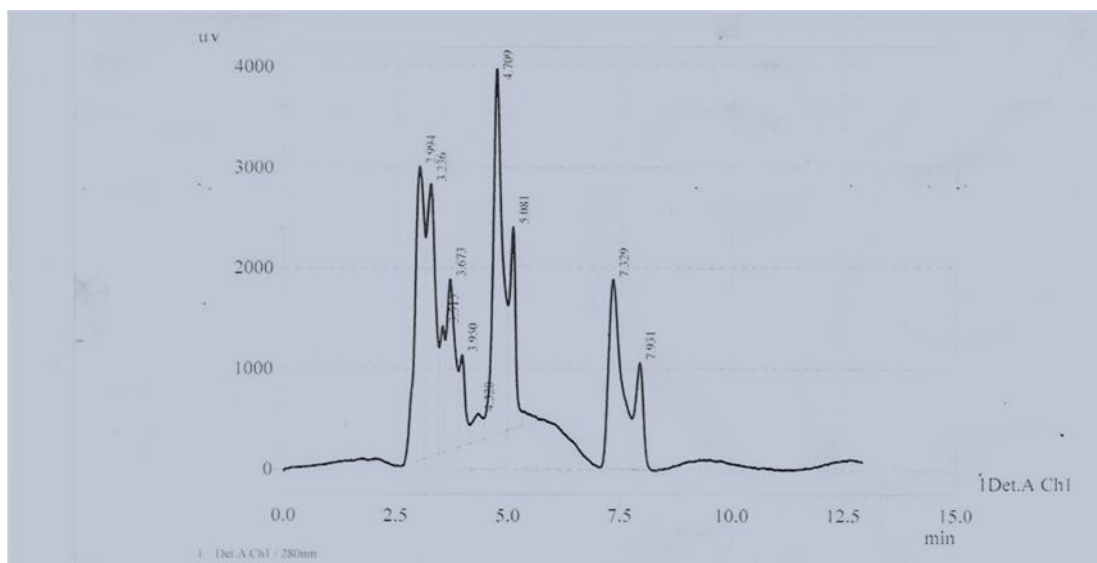


Fig.6 HPLC of 80% ethanolic extract

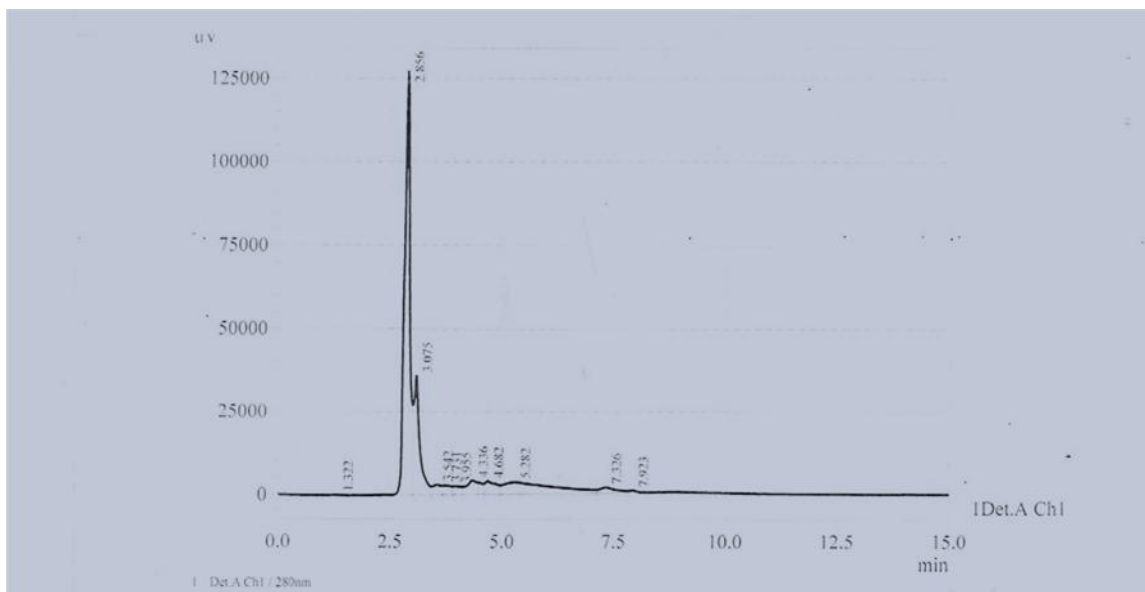
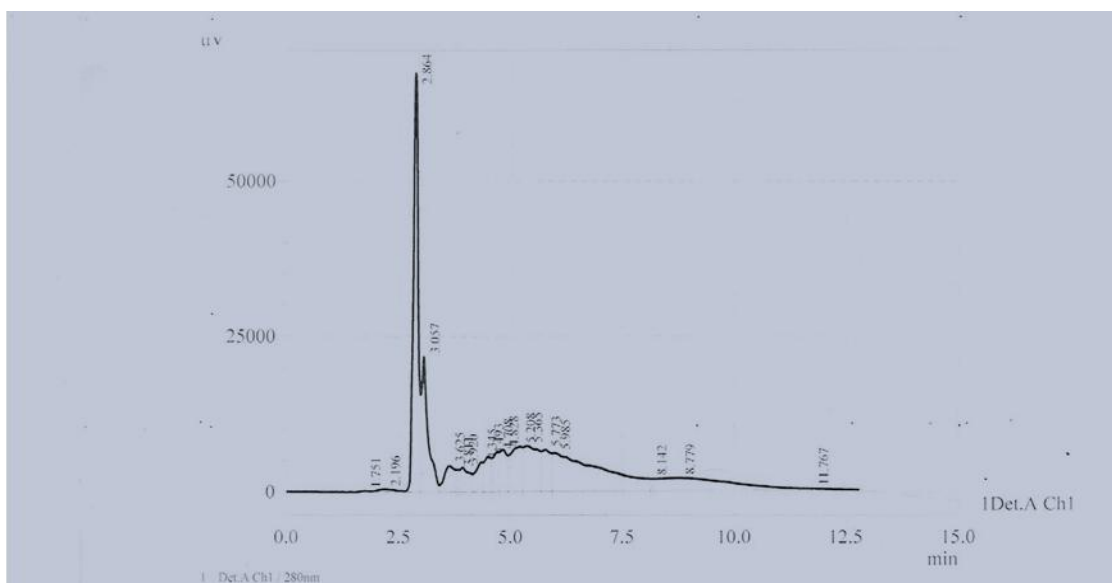


Fig.7 HPLC of water extract



Detection some flavonoids by HPLC

The results of three flavonoids detection using reversed phase HPLC are exhibited in the table 2 and shown in Figures 4, 5, 6 and 7). The results of (Rutin, Kaempferol and, Quercetin) concerning flavonoids constitution of corn silk extracts were: (0.012 mg/L) of Quercetin in 99% ethanolic extract, (0.13,

0.15 and, 0.11 mg/L) of (Rutin, Kaempferol, and Quercetin) respectively in 80% ethanolic extract, and (0.071, 0.091 mg/L) of (Rutin, and Kaempferol) respectively in aqueous extract. It is seen that 80% ETHANOL gives better and higher concentrations than others and it is suitable for all three types of flavonoids.

Urease inhibitory activity in *Klebsiella* Species

The inhibitory activity of Extracts (99.9%EE), (80%EE), and (AE) to *Klebsiella* species are shown in the table 3. It is noticed that all three extracts (99.9%EE), (80%EE), and (AE) show a potent urease inhibitory activity (IC₅₀ = 77.06 ± 1.19, 13.54 ± 4.02, and 35.93 ± 2.37 mg/L) respectively as compared with Thio urea which shows inhibitory of (IC₅₀ = 453.4 ± 1.84 mg/L).

The IC₅₀ Values of Extracts and the standard thiourea as urease inhibitor in *Klebsiella* species urease are shown in table 4.

Urease inhibitory activity in pure enzyme

Table 5 shows the pure inhibitory activity of the three extracts (99.9%EE), (80%EE), and (AE). It is noticed that all three extracts (99.9%EE), (80%EE), and (AE) show a potent urease inhibitory activity with (IC₅₀ = 402.8, 95.8, and 348 mg/L) respectively as compared with Thio urea which exhibits (IC₅₀ = 54077 mg/L).

The IC₅₀ Values of Extracts and the standard thiourea as urease inhibitor in pure urease enzyme are shown in table 6.

The crudes based flavonoids were extracted by using 80% ethanol, 99% ethanol, and water. Three of flavonoids: Rutin, Kaempferol, and Quercetin were detected using reversed phase HPLC. The 99% ethanol was able to extract only Quercetin; whereas, the Aqueous was able to extract Rutin and Kaempferol; but only 80% ethanol could extract such three flavonoids. It was found that water solvent gives higher yields than others; which can be considered as a factor causes solvent cost reduction. As well as, the three extracts of the corn silk were utilized in the inhibition of urease enzyme, it turned out

that 80% ethanolic extract exhibited a highest efficient inhibition of pure urease enzyme and *Klebsiella* species urease; as compared with 99% ethanol and the Aqueous.

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