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The antimicrobial, antioxidant, and in vivo hypoglycemic activities of the of the carob extract

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Abstract: *Ceratonia siliqua L. (carob) was found in Mediterranean region and many Arab countries and have many medical uses. The antimicrobial, antioxidant, cytotoxicity and hypoglycemic activities were determined. The extract of organic solvents, n-butanol, ethyl acetate, methanol and chloroform and the water extracts of Ceratonia siliqua leaves were evaluated. The microbial activity of* Ceratonia siliqua *leaves extract was evaluated against medically important bacterial and fungal strains. The antimicrobial activity was determined in the extracts using agar disc diffusion method. The antimicrobial activity of organic and aqueous extracts of* Ceratonia siliqua *against some Gram negative and positive bacteria was compared. Water extract showed moderate antibacterial and antifungal activities with MIC ranged from 25-50mg/ml and 50-70 mg/ml, respectively. The phenolic content of the leaves was determined. The antidiabetic and antioxidant effects of in diabetic male rats were determined. No toxicity was recorded for all tested concentration of water extract up to 400 mg/ml and LD50 was >400 mg/ml. Moreover, water extract of* Ceratonia siliqua *at 800 mg/kg decreased significantly the plasma glucose level (*$P \leq 0.05$*) in diabetic rats, and there is a considerable gain in body weight (*$P \leq 0.05$*) compared to the diabetic control group. In conclusion,* Ceratonia siliqua *leaves extract*

Keywords: *Ceratonia siliqua, bacteria, antimicrobial activity, cytotoxic activity, hypoglycemic, antioxidant*

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I. Introduction

In the last decade, plants are valuable sources of many secondary products used to keep human health. The evergreen perennial tree, *Ceratonia siliqua* L. (carob), is distributed in the Mediterranean region and attracted much attention due to economically importance. It belongs to the family Leguminosae (Kivçak and Mert, 2002) as widely planted an ornamental shade tree in kingdom Saudi Arabia and Australia (Esbenshade and Wilson, 1986). Leaves are used in folk medicine as antidiarrheal, diuretic pharmaceutical and cosmetic agents and chemical analysis indicated presence of tannins and polyphenols, thus human consumption is limited due to tannins which cause astringency (Custódio *et al*., 2011, Ashok and Upadhyaya, 2012).

The use of natural medicinal plant products as antimicrobial agents has gradually increased in India, Brazil and Arab countries and more than 80% of people used traditional plants instead of antibiotic (Ellof 1995, Nascimento *et al*., 2000, Aly *et al*., 2003) as the plants considered a source many drugs. Moreover, extracts of Carob pods were antihyperglycemic, antioxidant, immunomodulating and antiproliferative which may be attributed to plant phenolics, including flavonoids and phenylpropanoids (El Hajaji *et al*., 2010). *Ceratonia siliqua* leaves are rich in flavonoids and phenolic compounds and more than nine compounds were identified (Vaya and Mahmood, 2006) with antioxidant activity (Owen *et al*., 2003, Ayaz *et al*., 2007) which has strong DPPH radical scavenging activity (Papagiannopoulos *et al*., 2004).

The disorder Hyperglycemia is opposite to hypoglycemia and mean high blood sugar circulates in the blood plasma, between ~5.6 and ~7 mmol/l (100–126 mg/dl) while above 7 mmol/l (126 mg/dl) is generally mean diabetes but when the level exceed 7 mmol/l (125 mg/dl), organ damage was noted (American Diabetes Association guidelines, Capes *et al*., 2001). Levels of blood glucose above normal may have no symptoms while higher level have wide variety of complications like kidney, cardiovascular and neurological damages and long-term hyperglycemia lead to diabetic that need treatments by insulin or some plant extracts to maintaining blood glucose level like normal. The aqueous extract of *Spondias pinnata* (Anacardiaceae), *Kokoona zeylanica* (Celastraceae), *Syzygium caryophyllatum* (Myrtaceae), *Gmelina arborea* (Verbenaceae), aerial part extracts of *Scoparia dulcis* (Scrophulariaceae), *Sida alnifolia* (Malvaceae), leaf extract of *Coccinia grandis* (Cucurbitaceae) and root extract of *Languas galanga* (Zingiberaceae) possess potent acute antihyperglycaemic activity in alloxan induced diabetic rats (Attanayake *et al*., 2013). For better understanding, the extracts of carob should be studied for their activities, toxicities and efficiency. The present study aimed to determine the antimicrobial, antioxidant cytotoxic and antihyperglycemic properties of the water extract of *C. siliqua*.

II. Material and Methods:

Plant material and extract preparations

Ceratonia siliqua L. leaves were collected in July 2015 and a voucher specimen of *Ceratonia siliqua* was deposited in the herbarium of Biology Department, King Abdulaziz University. Plant extracts was prepared, 100 g of the air dried powdered leaves *C. siliqua* was extracted with *either* n- butanol, ethyl acetate, methanol, chloroform and hot water for 4 hours under shaking in a dark place To ensure complete extraction, the extraction was carried out 3 times and the obtained extracts were collected together. After extraction, the organic layer was collected and dried using rotary evaporator to dryness. The hot water extract was filtered through a mesh and a Whatman paper No. 2, and lyophilized to dryness. Each extract was dried, weighted, and dissolved in 2 ml dimethyl sulphoxide (DMSO), and antimicrobial activities were determined using agar well diffusion method. Leaves of *C. siliqua* were powdered, extracted, and dried and the diabetic rats received aqueous extract 500 mg/kg for 5 weeks..

Antimicrobial activities of the plant extracts

The tested Gram negative bacteria were *Escherichia coli* ATCC 29998, Enterobacter cloacae ATCC 13047, *Pseudomonas aeroginosa* ATCC 27853 and *Salmonella thyphimurium* CCM 5445 while the Gram positive were *Staphylococcus aureus* ATCC 6538P, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212. Candida albicans ATCC 10239 was used as yeast while *Aspergillus niger*, *Aspergillus clavatus* were used as test fungi. Agar well diffusion method was used to determine the antimicrobial activities. Sterile swaps was used to distribute 0.1 ml overnight cultures with $4x10^6$ cfu/ml of the tested bacterium and $6x10^4$ cfu/ml of the tested fungi on Petri dishes containing either Nutrient broth for bacteria or Sabouraud Dextrose agar for fungi. Using sterile cork-borer (7 mm), agar wells were created and filled with the tested organic or water extracts.

Toxicity of the tested plant extract

Toxicity was determined using brine shrimp lethality bioassay test and Artemia salina) assay. The cytotoxic activity of all extracts was compared to colchicine. Brine shrimp eggs (CA 94560 USA) was hatched in saline water after incubation for 2 days at room temperature. Different concentrations of the plant extracts (100- 1000 mg/ml) were tested in small dishes contained 10 larvae/plate. After 8 hr. of incubation, the surviving larva was counted for each plant extract concentration and LC_{50} was calculated.

Total phenolic content

Phenolic content of the plant extract was determined using Folin-Ciocalteu method and phosphomolybdic and phosphotungstic acids (Waterman and Mole, 1994). The absorbance at 764 nm of a mixture of 0.2 ml of the extract, 0.2 ml of Folin–Ciocalteu reagent and 0.3 ml of 25% Na_2CO_3, added after 8 min, was determined. The absorbance of all the samples was measured at 760 nm UV–Vis spectrophotometer and the results are expressed in mg of gallic acid equivalent per g of dry plant extract (mg garlic /g). The amount of phenolic content was calculated from gallic acid standard, carried from 0 to 500 mg/l, and the results were determined as gallic acid equivalents.

Determination of Antioxidant Capacity

Antioxidant Capacity (mg ascorbic acid/g dried sample) was determined, 2 ml of the plant extracts was added to 2 ml of 1 mM DPPH and the mixture was put for 60 min. in the dark, then the absorbance was determined at 517 nm (A_{517} nm). The results were shown as mg ascorbic acid/g dried sample. Each assay was carried out in triplicate.

Oxidation of Methyl linoleate

Antioxidant testing Methyl linoleate where plant extract was added to Methyl linoleate (0.2 g) in the dark at 40 °C and after 72 h of oxidation, 5 ml of 2,2,4-trimethylpentane (isooctane) was added and absorption at 234 nm was measured (Fischwick and Swoboda, 1977, Heinonen *et al*., 1998) as % of inhibition diene R-Tocopherol (20 ppm) was used as control.

Hyperglycemia in rats

Hyperglycemia in rats or diabetic was inducted as explained by Noaman *et al*. (2007). Rats were not received any food for 16 hours before intraperitoneal injection using 150 mg/kg of body weight of alloxan monohydrate. Alloxan monohydrate solution was prepared by dissolving alloxan monohydrate (Sigma Co., USA) in distilled water. Glucose levels of plasma were measured by the absorbance at 546 nm (A_{546}) using spectrophotometer (Baragob *et al*., 2014) and glucose concentration was determined as the following:

$$\text{Sample glucose conc. (mg/dl)} = \frac{\Delta\text{Abs. Sample}}{\Delta\text{Abs. Standard}} \times \text{Standard conc. (100 mg/dl)}$$

Where ΔA sample = (A sample - A blank), ΔA standard = (A standard - A blank)

Rat groups

In this study, the used randomly collected thirty adult male rats, weighing 200-250 g, were obtained from animal house of KFMR, KAU, Saudi Arabia and the rats were put in three groups (10 rats in each group). Animal plasma blood glucose levels of 200 -250 mg dl in fasting were studied as diabetic animals and were alienated in two groups. Negative control group or untreated rat group: 10 animals were used as control, Diabetic group: 10 diabetic animals stayed untreated. Treated group: 10 diabetic animals were treatment for 5 weeks with the prepared oral plant extract of carob leaves. All animals (the two diabetic rat groups and control rat group) were fed on basal diet and tap water. Finally, the rats in each group were fasted overnight, anaesthetized with diethyl ether and sacrificed. Blood samples were collected through heart puncture into a clean, dry centrifuge tube after 0 hr, 2 hrs, and 4 hrs time interval, allowed to coagulate at room temperature and centrifuged for 1 minutes at 3000 rpm to separate the serum which can kept frozen at -20˚C until analysis .

Statistical analysis

Experimental data were presented as mean ± SD (n = 10) and analyzed using one way analysis of variance to determine the significant differences between means using Statistical Packages for Social Science (SPSS) and mean differences at $P < 0.05$ considered significant.

III. Results and discussion

The antibacterial activity of *Ceratonia siliqua* leaves *extracts* using 4 organic solvents against some bacterial pathogens was determined (Table 1). It was found that the organic and water extracts of *Ceratonia siliqua* were significantly active against all tested bacteria compared to the activity of DMSO. The Ampicillin was used as positive control. The n-butanol extract was the most active extract against *Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis* and *Escherichia coli* with mean diameter of inhibition zone 25 mm. The water extract *Ceratonia* leaves extract showed moderate antibacterial activity against all tested bacteria with mean diameter of inhibition zone ranged from 11-14 mm. the lowest activity was for chloroform extract with mean diameter of inhibition zone ranged from 11-12 mm. The antifungal activity of the organic and water extract of *Ceratonia siliqua* was determined (Table 2). The extract of ethyl acetate and methanol showed the highest antifungal activity against *Aspergillus niger, A. clavatus, Candida albicans* and *C. tropicals.* The lowest antifungal activity was obtained with water extract with mean inhibition zone diameter of 10-11 mm. The MICs of the water extract if *Ceratonia siliqua* leaves was determined for some bacteria and fungi (Table 3). It was ranged from 25-55 mg/ml for bacteria and 50-70 mg/ml for fungi. The leaf extracts of carob (*Ceratonia siliqua*) showed antimicrobial activity *in vitro* against *Pectobacterium atrosepticum* (Meziani *et al*., 2015). The phenolic content of leaves extract was 1.83 ± 0.67 mg gallic acid/g ds and the Antioxidant Capacity was 0.8± 0.17 mg ascorbic acid/g dried sample and % of Inhibition of Methyl Linoleate Oxidation was 60%. No toxicity was recorded for all tested concentration of water extract up to 400 mg/ml and LD50 was >400 mg/ml. Moreover, water extract of *Ceratonia siliqua* at 800 mg/kg decreased significantly the plasma glucose level ($P \leq 0.05$) in diabetic rats, and there is a considerable gain in body weight ($P \leq 0.05$) compared to the diabetic control group (Table 5 and 6). Antioxidant such as carotene and vitamins C and E neutralize chemically active products of metabolism and reduced oxygen and free radicals damage the body. Primarily plant phenolics of the plant leaves are natural antioxidants. Several plant parts of vegetables and fruits like leaves, nuts, seeds, bark, and roots contained phenolics material that act as Antioxidant. Due to their potential antioxidant action, plant phenols and polyphenols, with their potential to act as antioxidants; play a major role in the prevention of various pathological conditions such as cancer, cardiovascular and neurodegenerative diseases believed to be associated with oxidative stress. Leaves are the most favorable storage sites for active ingredients. The extraction methods commonly employed in anti diabetic plant extraction are conventional methods involving solvents. However, the engineered extraction techniques such as supercritical extraction and microwave assisted extraction are gaining more attention due to the high efficiency of these techniques, and also because they produce a better yield of the active ingredients (Chan *et al*., 2012).

Research on diabetes treatment is gaining ground as the world population with diabetes is rising each year, and is expected to hit 439 million adults by 2030 (Shaw *et al*., 2010). The acting mechanisms are namely: alteration of glucose metabolism; hypolipidemic effect; pancreatic effect; antioxidative effect; diabetes complication treatment; and, insulin-like effect.

New anti-diabetic agents with no side-effects from plant extracts are needed and some plant parts are used by traditional people for their hypoglycemic effects *Heseachlamys sdulis Rosmarinus officinalis* and *Nelumbo nucifera* (Rodriguez *et al*., 1992 and Huralikuppi *et al*., 2011), and *Ceratonia siliqua* (Baragob *et al*., 2014). This study tried to determine the hypoglycemic effect of aqueous extract of the Carob in diabetic-treated rats at different concentrations at different time intervals. Bosch *et al*. reported the anti-diabetic effect of bitter melon in experimental animals. Similarly, Shahraki *et al*. (2007) observed that the serum glucose decreased significantly in diabetic rats after receiving 50 mg/kg *Teucrium polium* for a month Esmaeili and Yazdanparast showed a significant decrease in plasma glucose level in streptozotocin-induced hyperglycemic rats after 6 weeks of consecutive oral treatment with aqueous extract of *Teucrium polium* (Esmaeili *et al*., 2007). Alloxan is the most commonly used agents for the induction of diabetes in experimental animal models like rats (Baragob *et al*., 2014). In this study, rats were injected with alloxan solution (150 mg/kg body weight) to achieve hyperglycemia. The animals with more than 200 mg/dl plasma glucose level in fasting were considered diabetic for this study.

Baragob *et al*. (2014) found that *Balanites aegypticea* fruits, antispasmodic for stomach pain, can be used to treat diabetes. Our result shows clearly that the aqueous extract has hypoglycemic effects in alloxan-induced diabetic rats after 2 weeks treatment. These findings also support the use of this plant in traditional medicine as a hypoglycemic agent as reported in literature (Kamel *et al*., 1991).

Our results showed that the body weight of the diabetic rats was significantly raised when treated with 600 mg/kg aqueous extract (Group 3). These findings are in accordance with Ramesh and Pugalendi (2005) and Erenmemisoglu *et al*. (1997) studies who showed that *Teucrium polium* and *Rosmarinus officinalis* have hypoglycemic effects in normoglycemic and diabetic mice. The absence of weight loss or gain in weight, in treatment groups, probably may be due to some of the components of *Balanites aegypticea* aqueous extract, which may increase serum leptin levels (Ahren *et al*., 1997) and this increased serum leptin level ultimately rise circulating insulin as a direct correlation has been approved between leptin and insulin (Havel *et al*., 1996). The results also showed a very significant decrease in the plasma glucose level in group 3 immediately after the treatment, where the diabetic rats were treated with 600 mg/kg aqueous extract. As the time passed, the levels of plasma glucose levels increased although the differences were not much more significant at different time intervals compared to the diabetic control group. Similarly, the plasma glucose levels were also decreased in diabetic rat groups 3 and 4 where the diabetic rats were given 200 mg/kg and 400 mg/kg aqueous extract, respectively. However, this decrease in plasma glucose level was not so much significant compared to the diabetic control. One of the possible reasons for the increase in plasma glucose level with the passage of time may be the metabolism of the active ingredient of the extract responsible for the hypoglycemic effect.

The phytochemical investigation of the leaves of *Carob* revealed that it may contain rutin, interketones, and organic constituents, oils (volatile oils and fatty acids). It may also assume that the hypoglycemic effect of the aqueous extract may be attributed to its constituents such as rutin, saponins, and organic constituents. However, further studies are needed to find out the active ingredients of the extract. Complementary studies about the role of each active ingredient and its mechanism of action will be helpful to determine the role of each component in reducing plasma glucose levels. Further research is needed to know the histological changes in the pancreas and liver of the diabetic rats with or without aqueous extract. Similarly, either the lowering of plasma glucose level in treating animals is therapeutic or preventive after stopping the treatment dose or ingestion of the extract should be elucidated further in the near future.

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Table 1 : The antibacterial activity (diameter of inhibition zone, mm) of *Ceratonia siliqua* extracts using 4 organic solvents against some bacterial pathogens

Tested bacteria	Tested extract					Control Ampicillin (5µg/ml)
	n-Butanol	Ethyl acetate	Methanol	Chloroform	Water	
Staphylococcus aureus	25.1 ±2.1*	14.1 ±1.0*	16.0 ±1.9*	10.3 ±2.4*	11.2 ±2.0*	24.0±1.1
Staphylococcus epidermidis	25.4 ±1.0*	16.2 ±1.0*	18.0 ±3.1*	10.0±1.9*	14.0±1.3*	24.0**±1.2**
Enterococcus faecalis	25.9 ±1.7*	14.1 ±2.9*	15 ±2.1*	12.0 ±2.7*	14.3 ±1.7*	20.0±1.3
Escherichia coli	25.0±1.7*	14.9±2.4*	18.1±2.1*	10..0 ±2.0*	14..5 ±3.2*	20.0±1.2
Enterobacter cloacae	23.2±4.1*	10.5±2.9*	21.0±2.0*	12.6±2.3*	14.6±2.0*	20.0±15
Pseudomonas aeroginosa	19.0±1.6*	14.3±2.3*	17.0±1.0*	10.0±2.0*	12.0±1.0*	21.0±2.1
Salmonella thyphimurium	21.0±1.8*	11.1±2.9*	18.0±1.0*	11.0±3.1*	14.0±1.1*	21.0±1.9

*: Significant result compared to negative control (DMSO) at P<0.05.

Table 2 : The antifungal activity (diameter of inhibition zone, mm) of ***Ceratonia siliqua*** extracts using 4 organic solvents against some fungal pathogens

Tested fungi	Tested extract					Control Nystatin (5µg/ml)
	n-Butanol	Ethyl acetate	Methanol	Chloroform	Water	
Aspergillus niger	15.6±1.6*	20.9±1.9*	20.76±1.8*	16.53±2.9*	11.5±1.2*	24
Aspergillus clavatus	19.3±1.4*	19.66±1.5*	20.5±2.9*	15.0±1.0*	10.4±1.3*	22
Candida albicans	14.6±1.0*	17.66±1.0*	19.7±1.1*	14.0±1.0*	10.2±1.9*	21

Candida tropicals	15.6±1.6*	22.9±1.9*	20.76±1.8*	16.53±2.9*	10.13±1.9*	24

*: Significant result compared to negative control (DMSO) at P<0.05.

Table 3. MIC of the *Ceratonia siliqua* water extract for some bacterial and fungal pathogens

Tested bacteria	MIC (mg/ml)		Tested fungi	MIC (mg/ml)	
	Water extract	Control Ampicillin		Water extract	Control Ampicillin
Staphylococcus aureus	30±1.4	4.1 ±0.2	*Aspergillus niger*	70.3 ±8.4	5.0±0.1
Enterococcus faecalis	25±6.4	3.2 ±0.1	*Aspergillus clavatus*	70.0±8.9	6.0±0.4
Escherichia coli	50±5.0	4.1 ±0.9	*Candida albicans*	50.0 ±5.7	5.0±0.3
Enterobacter cloacae	30±5.6	5.9±04	*Candida tropicals*	60..0 ±5.0	12.0±2.2

Table 4. Total phenolics, antioxidant and toxicity of the water extract of *Ceratonia siliqua*

Tested extracts	Total phenolics (mg gallic acid/g dried sample)	Antioxidant Capacity (mg ascorbic acid/g dried sample)	Antioxidant Capacity (% of Inhibition of Methyl Linoleate Oxidation)	Toxicity (LD_{50}, µg/ml)	
				Water extract	Control
Water extract	1.83 ± 0.67	0.8± 0.17	60 %	>400	250

Table 5. Effect of administration of *Ceratonia siliqua* plant extract on the weight of normal and diabetic rats

Time (days)	Negative control (healthy)	Positive control (Diabetic)	Diabetic and treated with 600 mg/kg
0.0	223.41±8.27*	150.37±3.15	153.51±4.1
15	259.75±4.63*	141.92±5.83	225.18±2.12*
30	299.08±1.88*	114.87±8.98	220.08±0.78*

Values are expressed as means ± S.D. (n=10), *: Values are differing significantly compared to control diabetic rats at P<0.05.

Table 6. Effect of administration of plant extract on the sugar level of normal and diabetic rats

Time (hrs)	Negative control (healthy)	Positive control (Diabetic)	Diabetic and treated with 600 mg/kg
0.0	84.4±2.27*	200.37±7.19	120.5±4.54
2.0	129.75±5.63*	241.92±7.33	145.1±3.32*
4.0	176.08±9.08*	264.87±11.48	183.1±7.08*

Values are expressed as means ± S.D. (n=10), *: Values are differing significantly compared to control diabetic rats at P<0.05.

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