Pharmacogn J. 2022; 14(6):
A Multifaceted Journal in the field of Natural Products and Pharmacognosy
www.phcogj.com

Original Article

Chemical Profiling and *In Vitro* α-amylase Antidiabetic Assessment of *Carissa Macrocarpa* Flower Extract Cultivated in Saudi Arabia

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History

- Submission Date: 03-10-2022;
- Review completed: 13-10-2022;
- Accepted Date: 24-10-2022.

DOI : 10.5530/pj.2022.14.

Article Available online

http://www.phcogj.com/v14/i6

ABSTRACT

Carissa macrocarpa is commonly known as Natal plum. Its fruits are palatable and used in manufacture of jams while other parts of the plant are used in traditional medicine to treat various diseases. The main objective of current study was to screen the presence of and isolate the various phytochemicals applying standard procedures and to evaluate the *in vitro* antidiabetic activity using α-amylase inhibitory assay. The total methanol extract of flower (TMEF) of *Carissa macrocarpa* was subjected to several chromatographic procedures. Results demonstrated that TMEF of *Carissa macrocarpa* is characterized by the content of different constituents such as flavonoids, steroids, saponins, tannins and carbohydrates at different levels. Chromatographic isolation led to the isolation of kaempferol-3-O-robinobioside and caffeic acid, which were confirmed *via* using ^{1}H, ^{13}C, DEPT, COSY, HMQC and HMBC NMR spectroscopic analyses. TMEF exhibited α-amylase inhibitory activity with IC_{50} value of 65.4 µg/ml when compared to that of the acarbose (standard) (IC_{50} = 39.6 µg/ml). In conclusion, current investigation endorses the traditional use of *Carissa macrocarpa* as antidiabetic herb. Hence, the studied TMEF of *Carissa macrocarpa* may have the potential being nutraceuticals products for pharmaceutical applications as antidiabetic herbal remedy.

Key words: *Carissa macrocarpa*, Apocynaceae, α-amylase inhibitory, Antidiabetic, Caffeic acid.

INTRODUCTION

The genus *Carissa* is one of the most important members of family Apocynaceae. *Carissa* consists of evergreen species, like shrubs and small trees which are native to the subtropical and tropical regions of Africa and Asia.[1] The genus *Carissa* is reported to contain diversity of constituents including; phenoilics, flavonoids, lignans, sesquiterpenes, coumarins and tannins.[2] Pharmacological studies on the genus *Carissa* demonstrated numerous activities like antioxidant, anti-inflammatory, antipyretic, antihyperlipidemic, wound curative, antibacterial, hypoglycemic, anti-tumor and improvement of kidney and liver dysfunctions.[3] The genus comprises roughly 36 species.[4] *Carissa macrocarpa* (*C. macrocarpa*) (Synonym: *Carissa grandiflora*) member of family Apocynaceae, is ornamental shrub that grows worldwide including Saudi Arabia and is native plant from South Africa commonly known as Natal plum.[5] it has edible oval fruits which are delicious and traditionally used in manufacture of jams, desserts and yogurt.[6] while this plant is also used in traditional medicine for the treatment of diarrhea, cough, and sexually-transmitted diseases.[7] Previous studies reported the cytotoxic, antioxidant and antimicrobial activities of leaves, stems, and roots as a result of the availability of phenolic compounds including flavonoids and phenolic acids.[8] To the author's best knowledge, there is limited data found on the chemical composition , the isolation of bioactive metabolites and antioxidant of *C. macrocarpa* flowers. Only, few studies could be found regarding chemical composition of essential oils and extract of flowers and their anticancer antibacterial properties of Tunisian origin.[3,9] Therefore, the aim of the current study is to establish the chemical profile and isolation of major constituents of the hydromethanolic extract obtained from flowers of *C. macrocarpa* of Saudi origin, and further evaluation of *in vitro* antidiabetic activity using α-amylase inhibitory model.

MATERIALS AND METHODS

General

The instruments and chemicals were used in the current study: ^{1}H and ^{13}C-NMR spectra were obtained using Bruker Avance III spectrometer at 400 MHz for ^{1}H-NMR and 125 MHz for ^{13}C-NMR. Various stationary phases were used in this study including (Diaion HP-20, silica gel and reversed-phase silica gel). Methanol , n-hexane, chloroform, ethyl acetate and n-butanol were of analytical grades. Acarbose and α-amylase from porcine pancreas were purchased from Sigma Aldrich (ST. Louis. Mo, USA).

Plant material

Plant flowers of *C. macrocarpa* were collected from King Faisal University, Al-Hasa, Saudi Arabia. Flowers were carefully collected and separated and subjected to air-drying according to standard herbarium procedures. A voucher sample (01-13-Sept-CM) was kept in Department of Pharmaceutical Sciences, College of Clinical Pharmacy, King Faisal University.

Cite this article: Alghafli DA, Albahrani ZA, Alnasser FH, Alnajdi AI, Alanazi GM, Bursheed HA, et al. Chemical Profiling and *In Vitro* α-amylase Antidiabetic Assessment of *Carissa Macrocarpa* Flower Extract Cultivated in Saudi Arabia. Pharmacogn J. 2022;14(6):.

Extraction

Flowers (300 g) was thoroughly extracted three times (for 1 week) using 3 liters of 70% methanol, applying cold maceration method to avoid destruction of active constituents .[8,10] Methanol extracts were compiled and concentrated using rotary evaporator and then freeze-dried to yield the total methanol extract of flower (TMEF) (25 g), which were kept in freezer for the next steps. TMEF (5 g) was mixed with 500 mL of distilled water and fractionated with n-hexane, chloroform, ethyl acetate and n-butanol, successively for preliminary screening of phytoconstituents.

Phytochemical screening

Qualitative screening of available metabolites was carried out base on the standard protocols available in literature.[11,12]

Flavonoid

Part of corresponding extract (2 ml) was blended with 2 % NaOH (1ml). The production of intense yellow color and turned into colorless on addition of few drops of diluted acid, obviously refer to the presence of flavonoid.

Alkaloid

Part of corresponding extract (0.5.ml) was mixed with dilute HCl (1.5 ml) and followed by filtration. Few drops of Dragendorff's reagent was added to the filtrate and monitored. The presence of alkaloids is confirmed *via* the development of orange or orange-red precipitate.

Saponins

Part of corresponding extract (1 ml) was suspended in distilled H_2O (20 ml) and then the produced mixture was vigorously stirred. The formation of persistence froth for at least 15 min referred to the presence of saponins.

Steroid

Part of corresponding extract (0.5 ml) was dissolved in 5 ml $CHCl_3$ and few drops of acetic anhydride and concentrated H_2SO_4 were added from the side of the test tube. The upper yellow layer with green/blue color indicated the presence of steroids.

Tannins/Phenolics

About few drops of 5% $FeCl_3$ solution was added to 0.5 ml of corresponding extract gives intense blue-greenish indicating the presence of tannins/phenolics.

Glycosides

Part of corresponding extract (2 ml) was mixed Fehling's solution (A) and Fehling's solution (B) and reactants were heated on a water bath for about two minutes. After heating, it gives a brick-red color that indicated the presence of glycosides.

Anthraquinones

Part of corresponding extract (0.5 ml) was boiled with dilute H_2SO_4 then filtered and cooled. The filtrate was extracted with $CHCl_3$ and dilutes NH_3 solution was added to it. The aqueous NH_3 layer became pink to red due to the presence of anthraquinones derivatives.

Cardiac glycosides

Part of corresponding extract (10 ml) was mixed with (4 ml) of solution of glacial acetic acid and 1 drop of 2% $FeCl_3$ followed by 1 ml of concentrated H_2SO_4. A brown ring formed between the layers demonstrated the presence of cardiac glycosides.

Carbohydrates

Part of corresponding extract (2 ml) was mixed with a 10 ml Molisch reagent. Then, 2 ml concentrated H_2SO_4 was added from the side of the test tube. The formation of a violet ring at the intersection of two layers indicated the presence of carbohydrates.

Isolation of the flower major compounds

TMEF (20 g) was defatted with n-hexane t to give defatted TMEF (15 g) using the protocol of separation described by Khalil *et al*.[8] The defatted TMEF (15 g) was subjected to diaion HP-20 column chromatography (using 200 g of stationary phase) and the mobile phase was distilled H_2O (1 L) followed by methanol (2 L) to give water fraction (3 g) and methanol fraction (12 g). The methanol fraction (12.0 g) was subjected to silica gel column chromatography (using 150 g of silica gel as stationary phase and applying gradient elution using different compositions of chloroform and methanol) to yield three main fractions. Fraction 2 (6 g) was separated by reversed-phase silica gel column chromatography (100 g, of stationary phase applying gradient elution using different compositions of water and methanol] to yield five sub-fractions. The sub-fraction 2-3 (200.4 mg) was purified by repeated reversed-phase silica gel column chromatography to give 11 mg of compound 1 (cpd1). Similarly, the fraction 3 (4 g) was fractionated by repeated reversed-phase silica gel column chromatography to yield 7 mg of compound 2 (cpd2).

In vitro anti-diabetic (α-amylase inhibitory assay)

The mixture for assessment was designed to contain 200 µl of 0.02 M $NaPO_4$ buffer together with 20 µl of standard α-amylase enzyme and different range of concentrations (20-100 µg/ml) of TMEF. The mixture was kept for 10 min at room temperature followed by the addition of 200 µl substrate for enzyme (1% starch suspension) to all the vessels of reaction. Later after 2h, the reaction was ended by the addition of 400 µl of 3, 5 di-nitro salicylic acid color reagent. Followed by keeping the reaction tubes in boiling water bath for 5 minutes, then the vessels were let to get cooled at room temperature and diluted with 15 ml of distilled water. The absorbance of each reaction mixture was measured at 540 nm. The control reactions were also designed accordingly, but without addition of TMEF and were compared with the test samples containing different concentration of TMEF freshly prepared in DMSO. The results were indicated as % of inhibition of activity using the following formula:

$$Inhibition\ activity\ (\%) = \frac{Abs(control) - Abs\ (extract)}{Abs\ (control)} X100$$

Where; Abs (control) is the absorbance of the control reaction (containing all reagents except the test sample) and Abs (sample) is the absorbance of different plant extracts. The IC_{50} values (inhibitory concentration which will produce 50% inhibition of the enzyme activity) of TMEF was determined. Acarbose was applied as a positive control in the concentrations ranged from 20 to100µg.[13] All experiments were performed in triplicates.

RESULTS AND DISCUSSIONS

Phytochemical screening of different fractions of flower extract

The preliminary screening for various chemical constituents in different fractions of TMEF showed the presence of different chemical constituents such as flavonoids, saponins, steroids, anthraquinones, tannins/phenol, glycosides and carbohydrates at different levels in different fractions of the flower extract and the absence of alkaloids and cardiac glycosides, as expressed in Table 1.

Table 1: Preliminary phytochemical screening of various fractions of flower of *C. macrocarpa*.

Constituent/Fraction	nH	CH	EA	BT	AQ
Flavonoids Alkaline solution test	-	-	++	++	+
Alkaloids Dragendorff's reagent	-	-	-	-	-
Saponins Foam test	–	+	+	+	-
Steroids Liebermann-Burchard test	+	+	+	+	-
Tannins/Phenols 10% $FeCl_3$	-	-	++	++	+
Anthraquinones Borntrager's test	-	-	+	+	-
Cardiac glycosides Keller Killiani test	-	-	-	-	-
Glycosides Fehling's test	-	-	+	+	++
Carbohydrates Molisch's test	-	-	+	+	++

nH; n-hexane fraction, CH; chloroform fraction, EA; ethyl acetate fraction, BT; butanol fraction, AQ; remaining aqueous fraction, Presence in high amount (++), presence in moderate amount (+), absence (-).

Table 2: ^{1}H and ^{13}C NMR spectral data of kaempferol-3-O-robinobioside (Cpd1) compared to literature (Lit.) (DMSO at 400 and 100 MHz, respectively).

Position	Type	Chemical Shift δ (ppm)			
		^{1}H-NMR, (multiplicity), (J in Hz)		^{13}C-NMR	
		Lit.	Cpd1	Lit.	Cpd1
1	O	-	-		
2	C(quaternary)	-	-	156.5	158.5
3	C (quaternary)	-	-	133.3	135.8
4	C (quaternary)	-	-	177.5	179.5
5	C (quaternary)	-	-	161.2	161.7
6	CH	6.18, s	6.09, s	98.8	99.9
7	C (quaternary)	-	-	164.0	166.1
8	CH	6.40, s	6.28, s	93.0	95.0
9	C (quaternary)	-	-	156.5	159.4
10	C (quaternary)	-	-	103.8	103.6
1`	C (quaternary)	-	-	120.9	122.6
2`, 6`	CH	8.04, d, 8	7.98, d, 8.8	131.0	132.5
3`, 5`	CH	6.85, d, 8	6.78, d. 8.8	115.1	116.2
4`	C (quaternary)	-	-	160.0	162.9
1``	CH(galactosyl)	5.31, d, (7.0)	4.92, d, (7.8)	102.1	105.6
2``	CH	-	3.18-3.73, m	71.1	71.7
3``	CH	-	3.18-3.73, m	73.0	73.2
4``	CH	-	3.18-3.73, m	68.1	69.7
5``	CH	-	3.18-3.73, m	73.6	73.9
6``	CH_2	-	3.18-3.73, m)	65.4	67.4
1```	CH(rhamnosyl)	4.39, s	4.42, s	100.1	101.9
2```	CH	-	3.18-3.73, m	70.7	71.7
3```	CH	-	3.18-3.73, m	70.4	71.9
4```	CH	-	3.18-3.73, m	72.0	72.1
5```	CH	-	3.18-3.73 ,m	68.3	70.2
6```	CH_3	1.05, d, (6.0)	1.08, d, (6.2)	17.9	17.7

δ (ppm); delta scale (part per million), Lit.; literature, s; singlet, d; doublet, m; multiplet.

Isolation and identification of secondary metabolites

The TMEF of *C. macrocarpa* (20 g) was subjected to various and repeated techniques of chromatography to yield two pure compounds; kaempferol-3-O-robinobioside (Cpd1) (11 mg) (Figure 2A,) and caffeic acid (Cpd2) (7 mg) (Figure 2B). The structure was elucidated by inspection of 1D- and 2D-NMR spectroscopic data including; ^{1}H, ^{13}C, DEPT, COSY, HMQC and HMBC. Results were compared with those available in literature. This study represents the first report on the isolation of kaempferol-3-O-robinobioside (isolated previously from the leaves)[8] (table 2)[14] and caffeic acid (table 3) [15] from flowers of *C. macrocarpa* .

Table 3: ^{1} **H and** ^{13}**C NMR spectral data of caffeic acid (Cpd2) (CD3OD at 400 and 100 MHz, respectively) compared to literature (Lit.) (CD3OD at 600 and 150 MHz, respectively).**

Position	Type	Chemical Shift δ (ppm)			
		^{1}H-NMR, (multiplicity), (J in Hz)		^{13}C-NMR	
		Lit.	Cpd2	Lit.	Cpd2
1	C(quaternary)	-	-	127.82	127.85
2	CH	7.06, d, (2.04)	7.07 (br. s)	115.10	115.14
3	C(quaternary)	-	-	146.82	146.81
4	C(quaternary)	-	-	149.47	149.46
5	CH	6.80, d, (8.16)	6.79, d, (8.0)	116.51	116.53
6	CH	6.95, dd, (2.04, 8.16)	6.95, d, (8.12)	122.88	122.86
7	CH	7.55, d, (15.88)	7.55, d, (15.76)	147.06	147.03
8	CH	6.23, d, (15.88)	6.23, d, (15.8)	115.55	115.60
9	C(carboxylic acid)	-	-	171.08	171.06

δ (ppm); delta scale (part per million), Lit.; Literature, s; singlet, br.s; broad singlet, d; doublet.

Figure 1: Photograph of shrubs of *Carissa macrocarpa* in gardens of King Faisal University during the flowering stage.

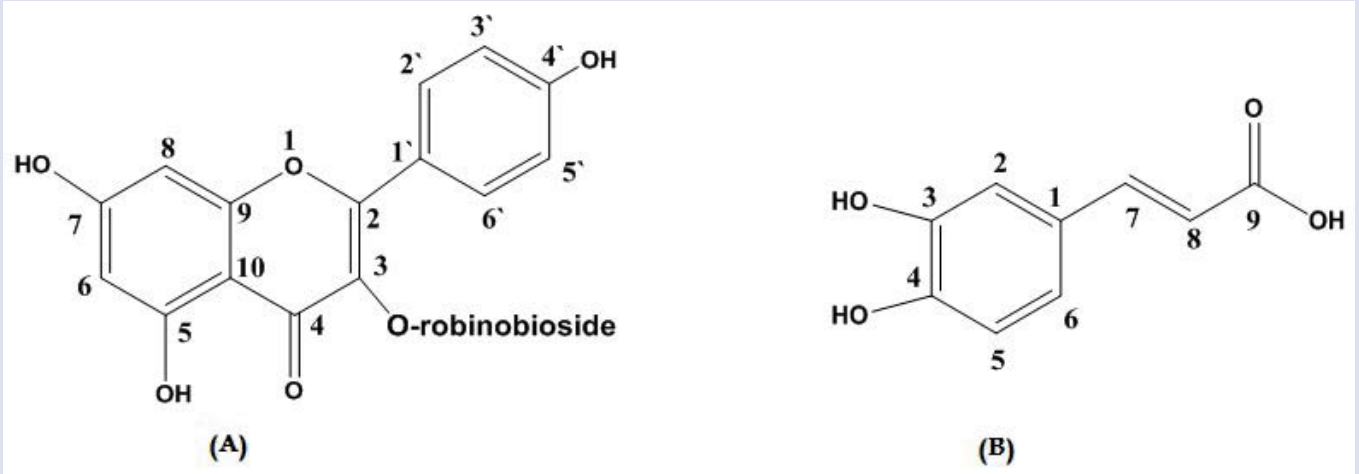

Figure 2: Structures of pure isolated compounds; kaempferol-3-O-robinobioside (A) and caffeic acid (B).

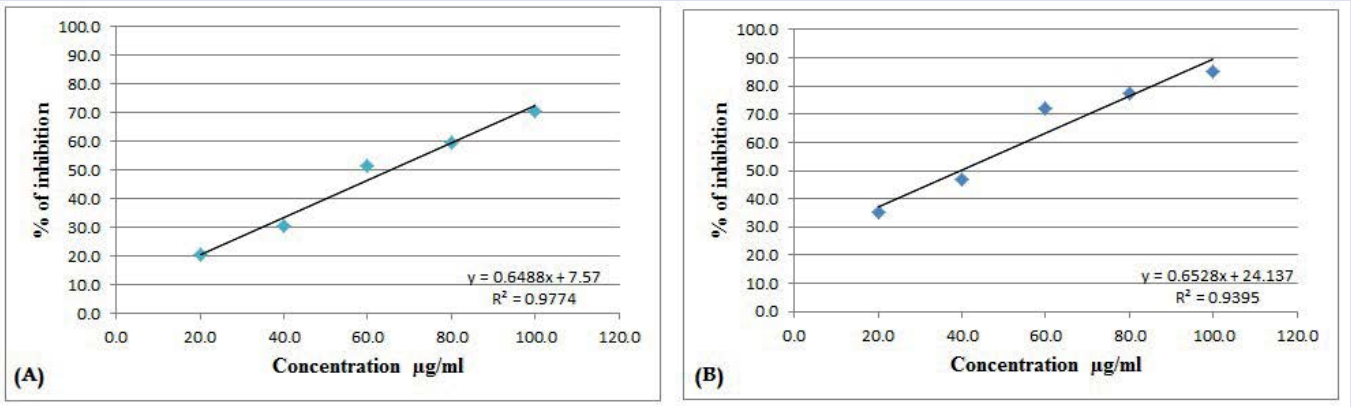

Figure 3: α- Amylase inhibitory effect of *C. macrocarpa*. Calibration curve for inhibitory effect TMEF of *C. macrocarpa* (A), Calibration curve for inhibitory effect of acarbose (standard)(B).

α-Amylase inhibitory activity

The *in vitro* α- amylase inhibitory assessment, demonstrated that TMEF of *C. macrocarpa,* has a possible of α- amylase inhibitory activity. α-Amylase inhibitory activities of TMEF and standard were compared based on the calculated IC_{50} values (Figure 3). Results demonstrated α-amylase inhibitory activity of TMEF of *C. macrocarpa* with a value of 65.4 µg/ml. Acarbose used as the positive standard showed IC_{50} value of 39.6 µg/ml under similar conditions. TMEF of *C. macrocarpa* showed promising result in α-amylase inhibition assay, suggesting that *TMEF of C. macrocarpa* might be effective in slowing down hydrolysis of polysaccharides like starch to glucose.

CONCLUSION

The present work demonstrated that flower of *C. macrocarpa* is characterized chemically by presence of flavonoids, saponins, steroids, anthraquinones, tannins/phenol, glycosides and carbohydrates and the absence of alkaloids and cardiac glycosides . Chromatographic procedures led to isolation of kaempferol-3-O-robinobioside and caffeic acid for the first time from its flower. *In vitro* antidiabetic examinations revealed a substantial α-amylase inhibitory of TMEF of *C. macrocarpa*. Confirmatory *in vivo* studies are recommended to evaluate the potential hypoglycemic effect of TMEF of *C. macrocarpa*.

ACKNOWLEDGEMENTS

This work was supported by the Deanship of Scientific Research, Vice Presidency for Graduate Studies and Scientific Research, King Faisal University, Saudi Arabia [Grant No. 1711], through its KFU Research Summer initiative.

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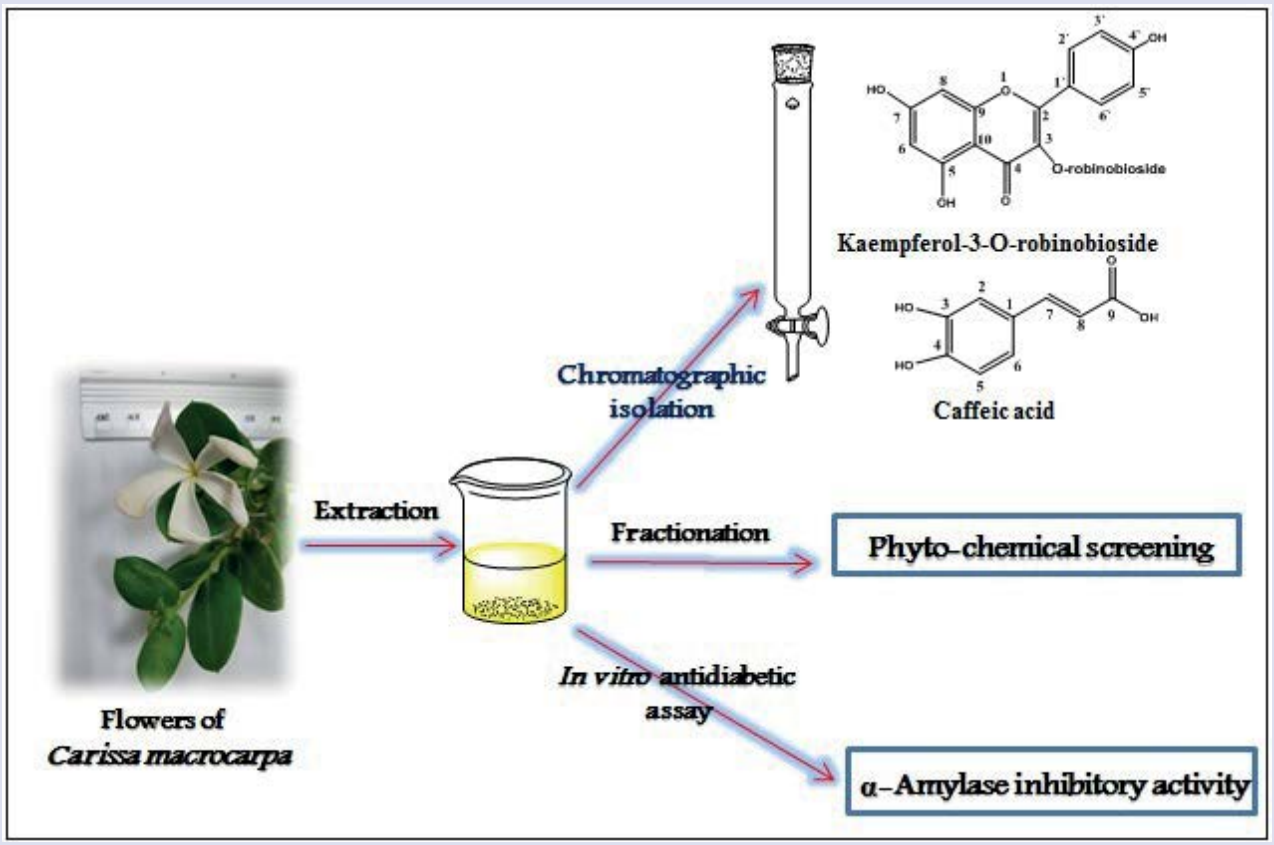
GRAPHICAL ABSTRACT



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Cite this article: Alghafli DA, Albahrani ZA, Alnasser FH, Alnajdi AI, Alanazi GM, Burshed HA, et al. Chemical Profiling and In Vitro α-amylase Antidiabetic Assessment of Carissa Macrocarpa Flower Extract Cultivated in Saudi Arabia. Pharmacogn J. 2022;14(6):.