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Polyphenolic Profiling and Analgesic Activity of *Citrullus lanatus* var. *citroides* Fruit

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ABSTRACT

Objectives: This study was designed for preliminary phytochemical screening of *Citrullus lanatus* var. *citroides* fruit pulp secondary metabolites. In addition, the polyphenolic compounds of the methanolic extract (ME) and its *n*-butanol soluble fraction (*n*-BF) were analyzed, and the *in vivo* analgesic activity of the richest polyphenolic fraction was evaluated. **Methods:** The ME was phytochemically screened for various secondary metabolites. Moreover, ME and *n*-BF polyphenolic profiling was performed using high performance liquid chromatography (HPLC) against external standards. The analgesic activity of the polyphenolic rich fraction (*n*-BF) was evaluated *in vivo* using acetic acid-induced abdominal writhing and hot plate tests. **Results:** Preliminary phytochemical screening revealed the presence of carbohydrate and /or glycosides, flavonoids, saponins, tannins, and unsaturated sterols and /or triterpenes, while alkaloids and/or compounds containing nitrogenous bases, anthraquinones, cardiac glycosides, and coumarins were absent. HPLC polyphenolic profiling showed the tentative identification of 11 phenolic compounds in ME (19.6959%), including 6 phenolic acids and 5 flavonoids, with gallic acid and rutin being the major identified ones; respectively. As well as, 17 phenolic compounds were tentatively identified in the *n*-BF (75.0771%), including 10 phenolic acids with chlorogenic acid as the major identified one, and 7 flavonoids with naringenin as the major one. The *n*-BF showed significant peripheral and central analgesic activity in a dose-dependent manner ($p < 0.05$), with *n*-BF at a dose of 1000 mg/kg being the most potent. **Conclusion:** The present study revealed that the *n*-BF was highly rich in phenolic compounds. These phenolic compounds were reported for the first time in *C. lanatus* var. *citroides*. The *n*-BF showed significant analgesic activity which was evaluated for the first time for the plant.

***Keywords**: Analgesic activity; Citrullus lanatus var. citroides; HPLC- Phytochemical screening; Polyphenols*

INTRODUCTION

Cucurbitaceae is a relatively large family comprising 95 genera and 1000 species, which are annual or perennial vines, sometimes woody, thorny shrubs or trees; distributed in tropical or subtropical areas, with few species spreading in temperate climate regions[1,2]. They are dicotyledons having familiar characters, including large leaves, creeping or climbing stems that typically have simple or branched tendrils, fleshy fruits called pepo that have leathery exocarps and contain multiple seeds, and woody rootstock. Three genera of Cucurbitaceae stand for the common name melons: *Cucumis*, *Citrullus*, and *Cucumeropsis*[3]. The genus *Citrullus* Schrad. ex Eckl et Zeyh. is native to Africa and comprises seven species[4]. *Citrullus lanatus* species is classified into two botanical varieties named: *C. lanatus* var. *lanatus* (Thunb.) Matsum. and Nakai, including the sweet cultivated watermelon and 'egusi' type, and *C. lanatus* var. *citroides* (L. H. Bailey) Mansf., comprising the "tsama" type and citroides group which is known as citron or preserving melon[5,6]. Citroides is a group of ancient cultigens in Southern Africa; it is called keeping melon because the fruit rind is used in preserves, jellies and conserves[7]. Morphologically citron melon has climbing or trailing stems 50–200 cm in length; fibrous roots; palmate green leaves having three to four pairs of lobes, mostly rounded with toothed margins and rough surface; solitary yellow flowers; green mottled fruits with light green and yellowish to whitish stripes having a smooth surface, round to oval in shape; rind is tough and strong, the mesocarp is white, dry and bitter, and having numerous seeds, ovoid to oblong-ovoid, tan or greenish and very tough[8,9].

Compared to other cucurbit species, citron melon has scant information on ethnomedicinal usage. Even though citron melon is native to Africa, commercial products are not widely developed, and the crop consumption is mostly neglected and underutilized in the region; this is ascribed to a lack of knowledge of its nutritional and phytochemical composition, in addition to the deficiency of dedicated research and development initiatives. Further research is therefore required for characterization and quantification of different phytochemical compounds. Citron melon's ethnomedical uses are likewise infrequently documented, and its usage as a medicinal plant is mostly unknown and unexplored[10].

Therefore, it was deemed of interest to explore the different phytochemical metabolites present in the plant fruit pulp and investigate the polyphenolic contents in the methanolic extract (ME) and its *n*-butanol soluble fraction (*n*-BF) using HPLC, together with an *in vivo* evaluation of the analgesic activity of the richest polyphenolic fraction.

MATERIALS AND METHODS

Plant material

Fruits of *Citrullus lanatus* var. *citroides* (L.H.Bailey) Mansf. (Family Cucurbitaceae) were collected from the field of the Arab Company for Pharmaceutical and Medicinal Plants (Mepaco), Anshas El Raml, Sharkia, Egypt, in June 2020. The plants were botanically identified and authenticated by Prof. Emad Farahat, Professor of Plant Ecology, Faculty of Science, Helwan University. Voucher specimens (8*Cla*1/2022) were kept at the herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Helwan University, Cairo, Egypt.

Experimental animals

Male and female Swiss albino mice (25-30 g) were sourced from the breeding unit of the Egyptian Organization of Biological Products and Vaccines located in Helwan, Egypt. The animals were housed in a breeding room with standard environmental controls and provided with *ad libitum* access to a standard pelleted diet and water.

Chemicals

Methanol (MeOH) and *n*-butanol (*n*- BuOH) were purchased from Piochem, Egypt. HPLC grades acetonitrile (Merck), authentic phenolic acids and authentic flavonoids were supplied by the National Research Centre, Giza, Egypt. Indomethacin and tramadol (Amadol® ampoule; 100mg/2 ml) were purchased from El-Nile Co. and Adwia Pharmaceuticals Co., Egypt, respectively.

Extraction and fractionation

Fruits' pulps of *C. lanatus* var. *citroides* (1.5 kg) were juiced by a blender and exhaustively extracted with 98 % MeOH under reflux. The extract was dried at 40°C under vacuum using rotary evaporator (Bűchi R-100, Switzerland), yielding the dry total extract (35 g). The methanolic extract was fractionated between distilled H_2O and *n*-BuOH saturated with water, yielding the *n*-BuOH soluble fraction (*n*-BF) (15 g) and the remaining aqueous residue.

Preliminary phytochemical screening

Methanolic extract of *Citrullus lanatus* var. *citroides* was phytochemically screened for different secondary metabolites, according to Morsy, 2014[11].

HPLC (High-Performance Liquid Chromatography) analysis

The polyphenolic contents of ME and *n*-BF were analyzed using the HPLC technique. HPLC analysis was carried out using an Agilent 1260 series.

The separation was carried out using Eclipse C18 column (4.6 mm x 250 mm i.d., 5 µm). The separation was carried out using Eclipse C18 column (4.6 mm x 250 mm i.d., 5 µm). Gradient elution of two solvents was used which consisted of water (A) and 0.05% trifluoroacetic acid in acetonitrile (B) at a flow rate of 0.9 ml/min. The gradient program was begun with 18% solvent B and increased gradually from 18 to 20% in the first 5 min, then increased gradually from 20 to 40% during the next three minutes. The concentration 40% B was kept constant for the following four minutes, after which the concentration of B was gradually decreased to 15%. The multi-wavelength detector was monitored at 280 nm. The injection volume was 5 µl for each of the sample solutions, and the column temperature was maintained at 40 °C.

Determination of acute oral toxicity (LD_{50})

Swiss mice were given oral doses of up to 5 g/kg of *C. lanatus* var. *citroides* *n*-BF, whereas the control group was given distilled water. The general behaviors and mortality rate of individual mice were assessed during a 24-hour period[12].

Analgesic activity

Acetic acid-induced abdominal writhing response in mice

The acetic acid-induced writhing test was used to assess the *n*-BF peripheral analgesic efficacy[13]. Thirty overnight starved mice divided into five groups, each of six were used for the study. The first and second groups were classified as control and standard drug groups, and received normal saline and indomethacin (10 mg/kg b.wt., i.p.), respectively. The remaining three groups were given *n*-BF orally in dosages of 250, 500, and 1000 mg/kg body weight. Injections of 0.6% v/v acetic acid (10 mL/kg b.wt.; i.p.) were given to all groups 60 minutes after the vehicle, standard drug or *n*-BF. After a latency of 5 min, the numbers of writhing were counted for 30 min. According to the following formula, the analgesic activity was expressed as a percentage of inhibition.

% Inhibition = Mean number of writhing (control) – Mean number of writhing (test)/ Mean number of writhing (control) x 100

Hot plate test in mice

According to Kakoti et al. (2013)[14], the hot plate method was used to assess the central analgesic activity of *n*-BF. Mice were allocated into five groups, control, standard (tramadol 10 mg/kg b.wt; i.p.), and three test groups received orally *n*-BF 250, 500 and 1000 mg/kg. Each mouse was individually placed into the Plexiglass cylinder on the hot plate surface (55 ± 1°C). The reaction time was determined before and after 60 minutes following the administration of vehicle, standard and *n*-BF. The reaction time is the number of minutes required for either jumping or paw licking. To determine the completion of the analgesic action and to avoid tissue injury, a cut-off period of 20 s was chosen.

Statistical analysis

Data were expressed as mean ± Standard Error (SE; SEM). GraphPad Prism, version 8 (GraphPad Software Inc., San Diego, California, USA) was used for statistical analysis and graphical representations, using a one-way analysis of variance (ANOVA), followed by a Tukey's test for determining the statistical significance between different groups. The value $p < 0.05$ was considered as significant.

RESULTS

Preliminary phytochemical screening

Methanolic extract of *C. lanatus* var. *citroides* fruit pulp was phytochemically screened for different metabolites, and the results are summarized in **Table 1**. Results revealed the presence of carbohydrate and /or glycosides, flavonoids, saponins, tannins and unsaturated sterols and /or triterpenes, and the absence of alkaloids and/or compounds containing nitrogenous bases, anthraquinones, cardiac glycoside, and coumarins.

Table 1. Preliminary phytochemical screening of *C. lanatus* var. *citroides* fruit pulp

Constituents	Results
Alkaloid and/or nitrogenous bases	-
Anthraquinones	-
Carbohydrate and/or glycosides	+
Cardiac glycosides	-
Coumarins	-
Flavonoids	+
Saponins	+
Tannins	+
Unsaturated sterols and/or triterpenes	+

N.B. (+) = Present, (-) = Absent

HPLC phenolic profiling

The phenolic compounds present in both ME and *n*-BF were tentatively identified by comparing their retention times with reference standards, while their concentrations were calculated by determining the percentage of the peak area, as shown in **Figures 1** and **2**, respectively. Results are compiled in **Table 2**.

Table 2. Methanolic extract and *n*-Butanol fraction of *C. lanatus* var. *citroides* fruit pulp HPLC phenolic profiling.

	Methanolic extract			*n*- Butanol fraction		
Identified phenolics	R_t (min)	% Area	Conc. (µg/g)	R_t (min)	% Area	Conc. (µg/g)
Gallic acid	3.335	8.7114	836.85	3.339	14.0807	3228.44
Chlorogenic acid	4.011	4.8092	909.31	4.013	22.8177	10297.35
Catechin	4.527	0.0000	0.00	4.717	0.1000	74.74
Methyl gallate	5.515	0.0000	0.00	5.454	0.0872	15.71
Caffeic acid	5.748	2.1692	229.46	5.754	19.3288	4880.01
Syringic acid	6.298	0.9306	112.77	6.322	5.6951	1647.14
Pyro catechol	6.770	1.1385	195.18	6.883	1.8592	760.73
Rutin	7.812	0.5449	90.63	7.577	2.3931	950.07
Ellagic acid	8.661	0.1953	77.33	8.652	0.8927	843.82
Coumaric acid	8.999	0.0000	0.00	9.008	0.1542	12.81
Vanillin	9.659	0.0000	0.00	9.722	0.1524	14.90
Ferulic acid	10.214	0.3877	27.76	10.117	0.6807	116.33
Naringenin	10.617	0.5316	62.85	10.599	2.9353	828.28
Daidzein	12.337	0.1002	7.89	12.326	1.3986	262.80
Quercetin	12.675	0.1773	26.34	12.688	1.8258	647.39
Cinnamic acid	14.013	0.0000	0.00	14.107	0.4903	29.84
Apigenin	14.476	0.0000	0.00	14.531	0.1853	33.54
Total flavonoids		2.4925%			10.6973%	
Total phenolic acids		17.2034%			64.1402%	
Total identified phenolics		19.6959%			75.0771%	

Acute toxicity study

Mice exhibited normal behavior and survived after receiving *n*-BF. As a result, the *n*-BF doses of 250, 500, and 1000 mg/kg were deemed to be safe.

Analgesic activity studies

Acetic acid-induced abdominal writhing test

This approach was utilized to evaluate the *n*-BF peripheral analgesic activity. It has been found that *n*-BF has a dose-dependent analgesic effect, as it significantly ($p < 0.05$) declined the number of mice writhing by 28.1, 42.4, and 60.5 %, at doses of 250, 500 and 1000 mg/kg b.wt, respectively, compared to the non-treated control group (**Figure 3A**). The standard treated group showed a significant decrease in writhing number ($p < 0.05$) with 70 %, as compared to the control group. Interestingly, the *n*-BF (1000 mg/kg) group showed a non-significant difference from the standard group.

Hot plate test in mice

Figure (**3B**, **C**) illustrates the central analgesic efficacy of *n*-BF. Compared to the control group, all the tested groups of *n*-BF and standard significantly ($p < 0.05$) prolonged the reaction time after 60 minutes of administration in a dose-dependent manner.

DISCUSSION

Limited studies have reported the isolation or identification of bioactive metabolites from *C. lanatus* var. *citroides*; and plant consumption is mostly neglected due to the lack of knowledge and research on its nutritional importance, phytochemical composition and medicinal use. This encouraged us to pay attention to this neglected valuable crop. The preliminary phytochemical screening of the fruit pulp constituents showed the presence of several phytochemical metabolites including polyphenolics (e.g. flavonoids).

Polyphenolic compounds are naturally occurring secondary bioactive metabolites from plant sources classified into several groups, including flavonoids and phenolic acids. They have several potential health benefits in addition to several therapeutic activities including anticancer, antioxidant, antimicrobial and antihypertensive activities, in addition to analgesic, antipyretic and anti-inflammatory activities[15- 17].

Since no previous studies reported the phenolic contents of *C. lanatus* var. *citroides* so, HPLC polyphenolic profiling was performed to reveal the

abundance of polyphenolic compounds in its fruit pulp. Seventeen phenolic compounds were tentatively identified in the *n*-BF, with a percentage of 75.0771%, while 11 phenolic compounds were tentatively identified in the ME with percentage of 19.6959%. All identified phenolic compounds were reported for the first time in *C. lanatus* var. *citroides*.

The polyphenolic richest tested sample (*n*-BF) was chosen for evaluation of its *in vivo* peripheral and central analgesic activities using chemical (acetic acid) and thermal stimuli methods.

The peripheral analgesic activity was evaluated using acetic acid induced writhing test, due to its sensitivity for detecting the antinociceptive effect at low doses of compounds. In addition, it is a recommended test for screening of the peripheral analgesic potentials[18]. The peritoneal cavity is irritated and stimulated by intraperitoneal injection of acetic acid, which results in the production and release of several endogenous inflammatory mediators including histamine, serotonin, bradykinin substance P, and PGs[19]. These cause chemical-induced visceral pain which is characterized by abdominal muscle tightness, as well as forelimb extension and body lengthening[20].

At all tested doses (250, 500 and 1000 mg/kg), the *n-BF* showed significant ($p < 0.05$) peripheral analgesic activity, as the number of writhing decreased with the respective values 28.1, 42.4 and 60.5 %, in comparison to the negative control. The observed results could be attributed to the high abundance of polyphenolic compounds, which are reported to have peripheral analgesic and anti-inflammatory activities[21].

The hot plate test, which was the second employed model, was used to detect the central antinociceptive mechanism and supra-spinal nociception[20,22]. Since mice's paws are extremely sensitive to heat at temperatures that are not harmful to their skin, the central antinociceptive mechanisms were noticed by placing the mice on a constant hot plate and watching their reactions. Those included jumping, withdrawal, and licking their paws. After the injection of centrally acting analgesics, the time until these reactions occur is prolonged[23]. This model was selected to evaluate the central analgesic potential due to its sensitivity to strong analgesics, need of short time and accuracy of measurements[20, 22].

The *n*-BF, at doses of 250, 500 and 1000 mg/kg body weight significantly ($p < 0.05$) elevated the pain threshold by increasing the reaction time after 60 min, as compared to the negative control, in a dose dependent manner. The central analgesic activity may be attributed to the presence of high concentrations of active polyphenols[24].

CONCLUSION

C. lanatus var. *citroides* fruit pulp revealed an abundance of polyphenolic compounds in the *n*-BF, where 17 phenolic compounds were tentatively identified for the first time from the plant. Additionally, *n*-BF showed significant peripheral and central analgesic activities, which was also reported for the first time for this plant.

Further studies are recommended for the isolation and identification of different secondary metabolites of the plant and for evaluating its other potential biological activities.

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Conflict of interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

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