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PHYTOCHEMICAL AND PHARMACOLOGICAL STUDIES ON *PULICARIA ORIENTALIS* JAUB & SP.

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تم فى هذا البحث تعريض الخلاصة الكحولية وخلاصة الكلوروفورم وخلات الاثيل للأجزاء الهوائية لنبات بوليكاريا اورينتالز (والمعروف بالعرار) النامى فى المملكة العربية السعودية لفحص أولى لمعرفة تأثيرها الخافض لنسبة السكر فى الدم وتأثيرها المضاد للالتهابات باستخدام الفورمالين لدى حيوانات التجارب. وقد أظهرت النتائج الأولية أن كل من الخلاصة الكحولية وخلاصة الكلوروفورم وخلات الاثيل قد أحدث انخفاضا معنويا على نسبة السكر فى الدم وتأثيرا واضحا كمضادات للالتهابات لدى حيوانات التجارب. وقد تم فصل ستة مركبات فلافونيدية من خلاصة الكلوروفورم ذات التأثير الفعال لنبات بوليكاريا اورينتالز وهذه المركبات هى: كامبفيرول-٣،٧-داى ميثيل ايثر، كامبفيرول-٣-ميثيل ايثر ، كورستين-٣-ميثيل ايثر ، ٢،٣-داى هيدروكامبفيرول ، كامبفيرول ، كورستين بالاضافه الى البيتاسيتوستيرول والبيتاسيتوستيرول جليكوزيد. كما تم فصل مركب فلافونى جليكوزيد من خلاصة خلات الاثيل وهو كورستين-٣-أ-جليكوزيد. وقد تم التعرف على هذه المركبات بواسطة دراسة الخواص الطبيعية والكيميائية والطيفية المختلفة لها ومقارنتها بعينات أصلية.

The alcoholic extract of the aerial parts of Pulicaria orientalis Jaub & Sp. (Arabic name Arar) growing in Saudi Arabia and its chloroform and ethyl acetate fractions were subjected to preliminary hypoglycemic and anti-inflammatory screening. The results showed that, the total alcoholic extract and its chloroform and ethyl acetate fractions showed a significant hypoglycemic effect in normal and glucose-loaded animals and a significant anti-inflammatory activity using formalin-induced edema.

From the pharmacologically active chloroform fraction of the total alcohol extract of the dried aerial part of Pulicaria orientalis six flavonoidal compounds were isolated and identified as kaempferol 3,7-dimethyl ether, kaempferol 3-methyl ether, quercetin-3-methyl ether, 2R,3R-dihydrokaempferol, kaempferol and quercetin in addition to β-sitosterol and β-sitosterol glucoside. Only one major compound was isolated from the ethyl acetate fraction and identified as quercetin-3-O-β-D-glucoside. The identification of the isolated compounds was carried out using different methods of physical, chemical and spectral analysis.

INTRODUCTION

The genus *Pulicaria* (Tribe Inuleae, F. Astraceae) is wildly spread in Egypt, Saudi Arabia, Qatar and other countries[1-5] and reported to have many folkloric and medicinal uses. *P. incisa* grown in Egypt showed hypoglycemic effect in rats.[6] The potential cancer chemopreventive and cytotoxic activities of *P. crispa* grown in Saudi Arabia[7] and the cytological effect of the same plant grown in Qatar[8] were studied. *P. dysenterica* was used for the treatment of hemorrhoids, abrasions and fissures[9-12] while *P. vulgaris* was used for treatment of papillomatosis.[13] *P. arabica* was reported to be used for the treatment of digestive disorders and its ethanolic extract exerted a significant decrease in serum glucose level and mild CNS depression. *P. undulata* was reported to be used as hemostryptic and counter-irritant and its alcohol extract showed a significant decrease in serum glucose level.[14-16] The genus *Pulicaria* is represented in Saudi Arabia by six species one of them is *Pulicaria orientalis.*[3]

The plants of the genus *Pulicaria* have been subjected to many phytochemical studies for their constituents specially flavonoids,[17-24] terpenoids[23-36] and other constituents.[37] Nothing

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was reported about the pharmacological action and constituents of *P. orientalis* in the available literature, so the study of this plant was thought to be interesting.

EXPERIMENTAL

General experimental procedures

Melting points were uncorrected and determined using electrothermal digital instrument, UV spectra were measured on Perkin-Elmer model 550 spectrophotometer. IR spectra were measured in Shimadzu Infrared 470 spectrophotometer using KBr. ^{1}H- and ^{13}C-NMR were measured in DMSO-d_6 at 400 MHz and 100 MHz respectively using Bruker AM-400 spectrometer, chemical shifts are given in δ values with TMS as internal standard. EIMS were recorded on a Jeol mass spectrometer 70 ev. For column chromatography, silica gel (E. Merck, 70-230 mesh), and for TLC, precoated silica gel plates (E. Merck) using solvent systems I-IV. Whatman No. 3 filter sheets was used for PPC using systems V and VI. The compositions of solvent systems used were as follow.

I- Chloroform - methanol (95:5)
II- Chloroform - methanol (90:10)
III- Chloroform - methanol (85:15)
IV- Chloroform - methanol -water (70:27:3)
V- Acetic acid - water (60:40)
VI- Butanol - acetic acid - water (4:1:2)

Spots were localized under UV light before and after spraying with 5% methanolic $AlCl_3$ or 10% methanolic H_2SO_4 and heating.

Plant material

The aerial parts of *Pulicaria orientalis* were collected from Saudi Arabia (Najd) at the end of March 1999. Prof. Dr. Dawoud M. H. Al-Eisawi, Prof. of Botany, University of Jordan, Amman, Jordan, kindly authenticated the plant.

Extraction of the plant material

1.87 kg of the air-dried aerial parts of *P. orientalis* was powdered and extracted with 80% ethanol by maceration and percolation for 24 h. This process was repeated for three successive times and the combined alcoholic extract was concentrated under reduced pressure (below 50°) till afforded a syrupy dark residue (82 g).

Fractionation of the total alcohol extract: Part of the dried alcohol extract (60 g) was mixed with water and successively extracted with chloroform (1.5 l x 4; fraction A) and ethyl acetate (1 l x 3; fraction B). The combined extract of each solvent was evaporated to dryness under reduced pressure to yield fraction A (32.5 g) and fraction B (18.5 g).

Preparation of the extracts for hypoglycemic and anti-inflammatory studies: specific weight of both the dried total extract and the chloroform and ethyl acetate fractions (4 g) were dissolved in distilled water by the aid of 1 % polyethylene glycol-400 (PEG-400) and the volume was completed to 100 ml with distilled water. A control solution was prepared using the same amount of PEG-400 (placebo).

Animals: Adult male albino mice obtained from Jordan University weighing 20-25 g were used. The animals were grouped in 8 mice group, the mice were housed at a standard condition in the experimental animal room for 7 days before use and fed laboratory diet *ad libitum* and allowed free access to water with 12 hours light and 12 hours dark cycle.

I- Hypoglycemic Study

a- Administration of the extracts and blood sampling: The total alcohol extract and the chloroform and ethyl acetate fractions were administered via stomach tube; the dose (400 mg/kg) was selected after preliminary behavior and acute toxicity test. Blood samples were withdrawn from the cavernous sinus with a capillary and the blood glucose level was determined (mg/dl).

b- Oral glucose tolerance test (O.G.T.T.): After overnight (18 hours) fasting, the glucose (2.25 g/kg body weight) [the dose was determined by trial and error using different glucose concentrations till the animals were hyperglycemic], solution was administered orally using stomach tube. Blood samples were collected at 15, 30, 45, 60, 90, 120 and 180 min. after the the glucose administration

c- **Effect of the plant extracts on the blood glucose level of fasting mice:** Both the total alcohol extract and the chloroform and ethyl acetate fractions solutions (400 mg/kg) were fed to overnight fasting mice at 0.0 min. and blood samples were drawn at 0.0, 30, 60, 90, 120 and 180 min. Control group received control solution (1% PEG-400) was used. Glipizide at a dose of 8 mg/kg was used as a positive control. The mice were kept unfed throughout the period of the experiment, the results were cited in Table 1.

d- **Effect on blood glucose level when the extracts were fed simultaneously with glucose:** Extracts solutions (400 mg/kg) with glucose solution (2.25 g/kg) were fed to overnight fasting mice at 0.0 min. and blood samples were drawn at 30, 60, 90, 120 and 180 minutes. Control group was fed with glucose solution (2.25 g/kg) was used; the results were cited in Table 2.

e- **Effect on blood glucose level when the extracts were fed 30 minutes before the glucose load:** Extracts solutions (400 mg/kg) were fed to overnight fasting mice at 0.0 min., after 30 min. the glucose solution was given to the mice. Blood samples were withdrawn at 30, 60, 90, 120 and 180 min. after glucose load, the results were cited in Table 3.

f- **Determination of blood glucose level:** Blood glucose levels were determined by the glucose oxidase method.[38]

g- **Statistical analysis:** All data were expressed as mean ±S.E. and students' t-test was used for the statistical analysis. P values of 0.05 or less were considered as significant.

II- Anti-inflammatory Study

Plant extracts (both of the total extract and the chloroform and ethyl acetate fractions) were administered orally once a day for four days via stomach tube at a dose 400 mg/kg. Sodium salicylate was used as a positive control at a dose 200 mg/kg and 1% PEG-400 solution was used as negative control.

Formalin-induced paw edema in mice: 0.05 ml of 3.5 % formalin solution in 0.9 % normal saline was injected into the right paw. The left paw injected by saline as a control. One hour after formalin injection, the inflammation was measured by cutting both feet at the level of the knee joint and comparing the weight of the right and left paws.

The increase in the paw weight was determined as:

$$I\% = \frac{Wr - Wl}{Wr} \times 100 \quad \text{where}$$

I % = increase in the paw weight.
Wr = weight of the right hind paw that received formalin.
Wl = weight of the left hind paw that received 0.9 % normal saline.

Tested extracts (total extract and chloroform and ethyl acetate fractions) and sodium salicylate were used one hour before formalin injection. The results were cited in Table 4.

III- Isolation of the constituents of the chloroform fraction

Fifteen grams of the dried chloroform fraction was fractionated over silica gel column chromatography (100x3 cm) using gradient elution with chloroform-ethanol. Fractions 100 each were collected and monitored with TLC, similar fractions were combined. Fractions 2-4 eluted with chloroform upon repeated crystallization afforded compound [C-**1,** 80 mg]. Fractions 6-8 eluted with chloroform-ethanol (98:2) afforded compound [C-**2**, 44 mg] while fractions 9-14 upon preparative PC using system IV afforded compounds [C-**3**, 54 mg] and [C-**4**, 35 mg]. Fractions 16-20 eluted with chloroform-ethanol (94:6) upon repeated crystallization afforded compound [C-**5**, 23 mg] while fractions 22-25 eluted with chloroform-ethanol (90:10) when subjected to PPC and repeated crystallization from methanol afforded compounds [C-**6,** 40 mg] and [C-**7**, 58 mg]. Fractions 31-36 eluted with chloroform-ethanol (88:12) gave compound [C-**8,** 112 mg].

Compound (C-1): colorless needle crystals, m.p 136-139°. It's IR spectrum was similar to that reported for β-sitosterol, further confirmation was carried out by mixed m.p and co-chromatography.

Table 1: Hypoglycemic effect of total alcohol extract and both of its chloroform and ethyl acetate fractions on normal mice.

Group No.	Zero M ± S.E.	30 min M ± S.E.	60 min M ± S.E.	120 min M ± S.E.	180 min M ± S.E.
Group I	68.8±5.7	90.5±10.5	63.3±3.7	60.3±3.5*	54.5±3.5**
Group II	69.8±2.8	88.8±4.5	56.2±1.8	58.8±1.3**	55.2±1.5***
Group III	73.5±3.8	96.6±4.5	64.2±1.9	61.0±1.2**	57.8±1.1***
Group IV	62.2±3.6	80.8±4.6	90.4±6.4	87.0±7.5	81.6±5.8
Group V	61.24±5.51	55.04±5.11*	51.08±4.88**	50.13±4.56***	-

Group I : Received the total alcohol extract (400 mg/ kg).
Group II : Received the chloroform fraction (400 mg/ kg).
Group III : Received the ethyl acetate fraction (400 mg/ kg).
Group IV : Received the negative control.
Group V : Received positive control.
These data represent the blood glucose level (mg/dl).
*P< 0.05; **P<0.01; ***P<0.001 (using students' t-test).

Table 2: Hypoglycemic effect of total alcohol extract and both of its chloroform and ethyl acetate fractions using O.G.T.T. when they are administered with glucose load concurrently.

Group No.	Zero M ± S.E.	30 min M ± S.E.	60 min M ± S.E.	90 min M ± S.E.	120 min M ± S.E.	180 min M ± S.E.
Group I	81.67±5.78	244.80±10.96	324.80± 12.74	210.80±11.94	113.82±8.14	--------
Group II	62.44±5.40	182.31±10.21	192.52 ±9.40*	112.16±9.4**	84.14±7.14**	----------
Group III	68.86±6.62	161.22±6.84	172.63±8.16**	130.31±6.22**	62.21±6.06***	64.19±5.85
Group IV	66.64±6.51	164.21±10.42	181.22±11.24*	138.84±13.28**	65.52 ±6.21**	52.81±4.82***

Group I : Received glucose load only (2.25 g/kg) .
Group II : Received glucose load and the total alcohol extract 400-mg/ kg.
Group III : Received glucose load and the chloroform fraction 400 mg/ kg.
Group IV : Received glucose load with ethyl acetate fraction 400 mg/ kg.
These data represent the blood glucose level (mg/dl).
*P< 0.05; **P<0.01; ***P<0.001 (using students' t-test).

Table 3: Hypoglycemic effect of total alcohol extract and both of its chloroform and ethyl acetate fractions when given 30 min after glucose load.

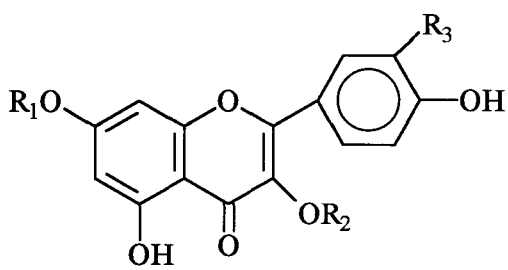
Group No.	Zero M ± S.E.	30 min M ± S.E.	60 min M ± S.E.	120 min M ± S.E.	180 min M ± S.E.
Group I	72.0±4.7	272.2±20.5	225.2±21.4	174.8±12.5	110.4±6.1
Group II	67.5±4.3	195.2±6.6*	143.7±3.9**	93.0±5.5**	75.5±5.9*
Group III	69.8±5.9	193.2±14.0*	115.5±8.8***	86.3±8.2***	74.3±3.2*
Group IV	68.8±5.1	199.6±15.6*	121.0±11.9***	88.6±7.6**	68.0±6.1**

Group I: Received glucose load only (2.25 g/kg) .
Group II: Received the total alcohol extract (400 mg/ kg) then after 30 min receive glucose load (2.25 g/kg).
Group III: Received chloroform fraction (400 mg/ kg) then after 30 min receive glucose load (2.25 g/kg).
Group IV: Received ethyl acetate fraction (400 mg/ kg) then after 30 min receive glucose load (2.25 g/kg).
These data represent the blood glucose level (mg/dl).
*P< 0.05; **P<0.01; ***P<0.001 (using students' t-test).

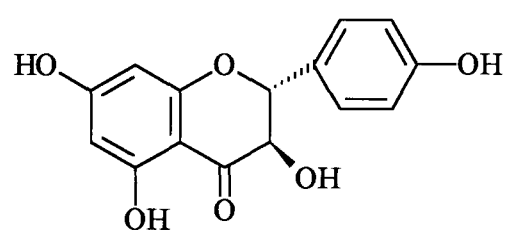
Table 4: Effect of the total alcohol extract and chloroform and ethyl acetate fractions on formalin-induced edema .

Group	Swelling of paws[+]	% of control
Control (PEG-400)	3.58 ± 0.35	100
Total extract	2.51 ± 0.27[*]	70
Chloroform fraction	1.98 ± 0.13[*]	55
Ethyl acetate fraction	2.23 ± 0.17[*]	62
Sodium salicylate	2.18 ± 0.11[*]	61

Values are expressed as mean ± S.E.
[+]Differences between weight of the right and left paw, g x 10^{-2}
[*]$P < 0.05$ compared to control using students' t-test.



Compound No.	R_1	R_2	R_3
C-2	CH_3	CH_3	H
C-3	H	CH_3	H
C-4	H	CH_3	OH
C-6	H	H	H
C-7	H	H	OH
E-1	H	Glucose	OH



Compound **C-5**

List of the isolated compounds from *Pulicaria orientalis* Jaub & Sp.

Compound (C-2): yellowish small needle crystals, m.p > 300°. EIMS m/z (% rel. int.) 314 (100), 313 (81), 296 (15), 285 (22), 167 (18) and 158 (12). The UV spectral data with different ionizing and complexing reagents are cited in Table 5 and 400 MHz ^{1}H NMR are listed in Table 6.

Compound (C-3): yellowish needle crystals, m.p 239-243°. EIMS m/z (% rel. int.) 300 (60), 299 (56), 283 (6), 282 (18) and 121 (100). The UV spectral data with different ionizing and complexing reagents are cited in Table 5 and 400 MHz ^{1}H-NMR (DMSO-d_6) were cited in Table 6.

Compound (C-4): yellowish needle crystals, m.p 215-219°. EIMS m/z (% rel. int.) 316 (100), 315 (78), 301 (7), 298 (14), 287 (10), 153 (22), 137 (48) and 109 (15). The UV spectra with different ionizing and complexing reagents were cited in Table 5 and 400 MHz ^{1}H-NMR (DMSO-d_6) were cited in Table 6. 100 MHz ^{13}C-NMR (DMSO-d_6): δ 156.02 (C-2), 137.99 (C-3), 178.21 (C-4), 161.64 (C-5), 98.98 (C-6), 164.75 (C-7), 93.95 (C-8), 156.60 (C-9), 104.38 (C-10), 121.03 (C-1`), 116.04 (C-2`), 145.62 (C-3`), 148.98 (C-4`), 115.89 (C-5`), 120.98 (C-6`) and 59.86 (OCH_3).

Compound (C-5): needle crystals, m.p 225-227°. EIMS m/z (% rel. int.) 288 (22), 153 (100), 152 (70), 136 (30), 134 (44) and 107 (31). The UV spectra with different ionizing and complexing reagents are cited in Table 5. IR ν^{KBr} 3450-3350 (OH), 1635 (γ-pyrane), 1610,

Table 5: UV Spectral data of the isolated flavonoids with different ionizing and complexing reagents.

Reagent compound	MeOH	NaOMe	$AlCl_3$	$AlCl_3$+HCl	NaOAc	NaOAc + H_3BO_3
C-2	256 266 295 348	256 266 298 396	276 299 350 398	277 307 346 398	261 268 298 389	269 301 351
C-3	256 270 296 355	256 275 354 398	272 302 353 399	277 303 354 399	273 303 328 375	267 304 330 354
C-4	255 267 295 356	273 297 326 406	275 305 338 438	275 305 365 403	276 331 389	269 301 321 365
C-5	229 292 325	227 246 324	228 295 350	227 304 320	227 279 329	228 297 321
C-6	257 268 293 370	277 294 412 dec.	272 302 376 428	271 313 328	277 291 330 394	265 273 292 378
C-7	255 267 295 371	270 298 415	275 299 335 446	267 277 298 405	264 279 297 410	259 301 390
E-1	254 305 358	271 327 410	272 301 329 433	272 297 353 402	273 322 383	259 379

Table 6: ^{1}H-NMR spectral data of the isolated flavonoids (DMSO-d_6).

Comp No.	H-6 δ (*J* Hz)	H-8 δ (*J* Hz)	H-2` δ (*J* Hz)	H-3` δ (*J* Hz)	H-5` δ (*J* Hz)	H-6` δ (*J* Hz)	OCH_3 or sugar δ (*J* Hz)
C-2	6.19, d (2.2)	6.45, d (2.2)	7.98, dd (8.6,2.2)	6.89, dd (8.6,2.2)	6.89, dd (8.6,2.2)	7.98, dd (8.6,2.2)	3.83, s 3.80, s
C-3	6.16, d (2.0)	6.40, d (2.0)	7.93, d (8.5)	6.95, d (8.5)	6.95, d (8.5)	7.93, d (8.5)	3.74, s
C-4	6.18, d (2.0)	6.38, d (2.0)	7.52, d (2.1)	---------	6.92, d (8.6)	7.41, dd (8.6, 2.1)	3.79, s
C-6	6.36, d (2.0)	6.55, d (2.0)	8.18, dd (8.8,2.0)	6.97, dd (8.8,2.0)	6.97, dd (8.8,2.0)	8.18, dd (8.8,2.0)	--------
C-7	6.28, d (2.2)	6.46,d (2.2)	7.75, d (2.0)	-----	7.16,d (8.5)	7.58,dd (8.5,2.0)	———
E-1	6.22, d (2.0)	6.38, d (2.0)	7.51, d (2.1)	-------	6.78, d (8.5)	7.65, dd (8.5, 2.1)	5.21, d (7.6) and 3.03-3.98

1530 (aromatic), 1255, 884 and 748 cm^{-1}. 400 MHz ^{1}H-NMR (DMSO-d_6): δ 4.54 (1H,dd, *J*= 11.3 and 7.0 Hz, H-3), 5.06 (1H, d, *J*= 11.3 Hz, H-2), 6.02 (1 H, d, *J*= 2.1 Hz, H-6), 6.08 (1H, d, *J*= 2.1 Hz, H-8), 6.86 (2H,d, *J*= 8.8 Hz, H-3` and H-5`), 7.38 (2H, d, *J*= 8.8, H-2` and 6`), 5.78 (1H, d, *J*= 7.0 Hz, C-3 OH), 7.55 (1H, br.s, C-7 OH), 9.55 (1H, br.s, C-4`OH) and 11.86 (1 H, br.s, C-5 OH).

Compound (C-6): yellow needles, m.p 285-286°. EIMS (% rel. int.) 286 (72), 258 (52), 153 (10), 152 (5) and 121 (10). The UV spectra with different ionizing and complexing reagents were cited in Table 5 and 400 MHz ^{1}H-NMR (DMSO-d_6) were cited in Table 6.

Compound (C-7): golden yellow needles, m.p > 300°. EIMS m/z (% rel. int.) 302 (100), 286 (88), 279 (18), 273 (15), 228 (10), 153 (12), 138 (13), 137 (35) and 121 (15). The UV spectra with different ionizing and complexing reagents are cited in Table 5 and 400 MHz ^{1}H (DMSO-d_6) as cited in Table 6.

Compound (C-8): small needle crystals (chloroform-methanol 1:1), m.p 277-278°. It was identified as *β*-sitosterol glucoside by m.p, mixed m.p, and co-chromatography with authentic sample (system III). Acid hydrolysis gave a sugar identified as glucose (PC using system V) and aglycone identified as β-sitosterol (m.p, mixed m.p and co-chromatography).

Isolation of Compound (E-1) from ethyl acetate fraction: 5 g of the ethyl acetate fraction were chromatographed over silica gel CC, using gradient chloroform-ethanol for elution. Fractions each 300 ml were collected and monitored using TLC silica gel (system VI). Fractions 28-34 eluted with chloroform-ethanol (80:20) contain a major spot, upon repeated crystallization from methanol it gave compound **(E-1)**.

Compound (E-1): obtained as yellow powder, m.p 206-209°. The UV data with different ionizing and complexing reagents were cited in Table 5 and its ^{1}H-NMR (DMSO-d_6) were cited in Table 6.

Acid hydrolysis: about 10 mg of compound [**E-1**] in 10 ml methanol were added to an equal volume of N/2 H_2SO_4 and refluxed in a water bath for 2 hours. The hydrolysate was extracted with ether (3x50 ml). The combined ethereal extract was dried over anhydrous Na_2SO_4 and the residue was crystallized from methanol to give the aglycone. The mother liquor (containing the sugar) was neutralized with barium carbonate, filtered and the filtrate was concentrated under reduced pressure. The residue was subjected to PC using Whatman No. 1 and solvent system VI. The sugar was identified as glucose. The obtained aglycone was identified as quercetin (m.p, mixed m.p and co-chromatography with authentic sample).

RESULTS AND DISCUSSION

I- Results of hypoglycemic activity

a- Effect on normal mice

All the tested materials showed a non-significant hyperglycemic effect on normal mice after 30 min. and this may be due to the presence of soluble sugars or transient hyperglycemic agents. After 2 hours all the tested materials showed a significant hypoglycemic effect and this effect was continued for more one-hour (Table 1).

b- Effect on glucose loaded mice

The O.G.T.T. showed the highest blood glucose level after 30 min. (Table 2). When the total extract and its fractions were given concurrently with the glucose load at a dose 400 mg/kg, a significant hypoglycemic effect was obtained after 1 hour and this continued for more two hours (Table 2), this may be due to non-insulin mechanism of the hypoglycemic effect. But when the tested materials was administered 30 min. after the glucose load, a significant decrease in the blood glucose level was observed after 1 hour, and this effect was continued through all time of the experiment, and this may suggest an insulin like mechanism of the tested materials. Since, the tested extracts showed a decrease in blood sugar level in both experiments, this might suggest a dual action of the constituents of these extracts.

II- Results of the anti-inflammatory study

Both of the total alcoholic extract and its chloroform and ethyl acetate fractions showed a significant anti-inflammatory activity, the total alcohol extract showed a significant reduction (30%) comparing with positive control. The chloroform fraction showed a reduction in edema more than the positive control, while the ethyl acetate fraction showed a reduction comparable with that of the positive control (Table 4). The edema and pain produced by formalin are mediated by substance P and bradykinin in the early phase, followed by tissue mediated response induced by histamine, 5HT, prostaglandins and bradykinins.[39] Thus, the anti-inflammatory activity of the total extract and its chloroform and ethyl acetate fractions may be attributed to inhibition of one or some of these inflammatory mediators.[40]

III- Identification of the isolated compounds

From the chloroform fraction of alcohol extract of *P. orientalis* six flavonoidal aglycones were isolated together with β-sitosterol and β-sitosterol glucoside, while from the ethyl acetate fraction, one flavonoidal glycoside was isolated. The identification of the isolated flavonoids was carried out according to the standard methods of identification.[41-46]

Compound C-1: (β-sitosterol)

The physical and chemical characters of compound **C-1** are similar to reported for β-sitosterol and this was confirmed by mixed m.p and co-chromatography.

Compound C-2: (Kaempferol-3,7-dimethyl ether)

The UV spectral data with different ionizing and complexing reagents (Table 5) indicated that this compound is a flavonol with free hydroxyl groups at C-5 and C-4` and blocked hydroxyl groups at C-3 and C-7 (purple color under UV light).[41] The presence of free hydroxyl group at C-5 was evident, since the compound appears as a purple spot on a paper chromatography when viewed in UV light (at 366 nm),[41] while the presence of C-4` hydroxyl group was confirmed by a band I bathochromic shift with increase in intensity with NaOMe relative to band I in MeOH.[41] EIMS showed M^+ at m/z 314 consistent with the molecular formula $C_{17}H_{14}O_6$ for flavonol having two methoxy groups, other significant peaks m/z at 313 $[M-1]^+$, 296 $[M-H_2O]^+$ in addition to other fragments characteristic for methyoxy flavonols.[41] The ^{1}H-NMR spectral data (Table 6) showed typical pattern of ring B characteristic for kaempferol, two *meta*-coupled protons for H-6 and H-8 of ring A, with additional two methoxy groups. From these spectral data (UV, MS and ^{1}H-NMR) compound **(C-2)** was identified as kaempferol-3,7-dimethyl ether.

Compound C-3: (Kaempferol-3-methyl ether)

The UV spectral data with different ionizing and complexing reagents showed the presence of C-7 and C-4` hydroxyls in a flavonol structure. Again the presence of free hydroxyl group at C-5 was evident, since the compound appears as a purple spot on a paper chromatography when viewed in UV light (at 366 nm),[41] while the presence of C-4` hydroxyl group was confirmed by a band I bathochromic shift with increase in intensity with NaOMe relative to band I in MeOH[41] and the presence of C-7 hydroxyl group was evidence d from band II bathochromic shift with NaOAc relative to band II in MeOH.[41] The ^{1}H-NMR (Table 6) showed a typical pattern of ring B, two *meta* coupled protons for H-6 and H-8 characteristic for kaempferol[41] and one methoxy signal assigned for 3-OCH_3. The MS exhibited M^+ at m/z 300 for the molecular formula $C_{16}H_{12}O_6$ with the other fragments characteristic for flavonol with three hydroxyl and one methoxyl groups.[41] From the entire above mentioned data compound **(C-3)** was identified as kaempferol-3-methyl ether (isokaempferide).

Compound C-4: (Quercetin 3-methyl ether)

The UV spectral data with different ionizing and complexing reagents (Table 5) showed the flavonol structure with methylted hydroxyl at C-3 (purple color under UV light) and free hydroxyls at C-7, C-3` and C-4`. The ^{1}H-NMR (Table 6) showed the presence of one methoxyl group (at δ 3.79) and a typical pattern similar to quercetin. Again, the presence of C-7 hydroxyl was deduced from the bathochromic shift in band II with NaOAc in the UV spectra, while the presence of C-3 hydroxyl together with the *ortho*-dihydroxyl groups in the ring B

was deduced from the high bathochromic shift in band I with $AlCl_3$ relative to band I in MeOH which was decreased after addition of HCl[41] (Table 5), and this was confirmed from the purple color of the spot under UV lamp. The EIMS showed m/z 316 calculated for $C_{16}H_{12}O_7$ and the ^{13}C-NMR showed the presence of sixteen carbon signals supported the molecular formula. The ^{13}C-NMR data are identical to those reported for quercetin 3-methyl ether[44] and this confirmed that compound (**C-4**) is quercetin-3-methyl ether.

Compound C-5: (2R,3R-dihydrokaempferol)

Obtained as needle crystals, m.p 225-227°, it gave a yellow color with alkali which suggest its phenolic nature. It exhibited UV absorption at λ_{max} 292 and 325 nm (shoulder) suggesting an isoflavone, a flavanol or flavanone.[46] The ^{1}H-NMR of compound (**C-5**) indicated its flavanol nature due to the presence of two doublets at δ 4.54 and 5.06 (AB pattern) characteristic for *trans* H-2/H-3 vicinal protons (J= 11.3 Hz) in the flavanols[45] and the UV λ_{max} at 292 and 325 nm were closely related to dihydroflavonol.[41] A bathochromic shift (25 nm) in the UV spectrum with $AlCl_3$ (Table 5), and IR absorption at 1635 cm^{-1} indicated the presence of a chelated hydroxyl *peri* to carbonyl. The ^{1}H-NMR showed the presence of four exchangeable protons (disappeared after addition of D_2O) at δ 5.78, 7.55, 9.55 and 11.86. ^{1}H-NMR also showed the presence of 1,4-disubstituted aromatic ring (B-ring). The IR absorption at 1635 cm^{-1} indicated the presence of a chelated hydroxyl group *peri* to the carbonyl group and this together with the ^{1}H-NMR signal at δ 11.87 attributed to C-5 hydroxyl group.[43,46] The MS showed M^+ at m/z 288 calculated for $C_{15}H_{12}O_6$ with the other fragments that confirmed the structure of compound C-5 as 5,7,4`-trihydroxy dihydroflavonol. Dihydroflavonols can occur in four sterioisomeric forms, owing to the asymmetric carbons C-2 and C-3, but, in nature, they occur predominantly as the 2,3-*trans* isomers with the C-2 phenyl and C-3 hydroxyl group in equatorial position.[47] The majority have the absolute configuration (2R,3R). Reports of 2,3-*cis* isomers are rare. The relative thermodynamic stability of *cis*- and *trans*-dihydroflavonols was discussed and the authors suggested that, owing to the hydrogen bonding between the carbonyl group and the equatorial 3-hydroxyl group, the 2,3-*trans* isomer should be favored. So, compound C-5 was identified as 2R,3R-dihydrokaempferol.

Compound C-6: (Kaempferol)

The physical characters and spectral data (UV and ^{1}H-NMR) of compound (**C-6**) [Tables 5 and 6] are identical to those reported for kaempferol,[41] further confirmation was carried by co-chromatography and mixed m.p using authentic sample.

Compound C-7: (Quercetin)

The physical characters and spectral data (UV and ^{1}H-NMR) of compound (**C-7**) [Tables 5 and 6] are identical to those reported for quercetin,[41] further confirmation was carried by co-chromatography using authentic sample.

Compound C-8: (β-sitosterol glucoside)

The physical and chemical characters of compound (**C-8**) are similar to those reported for β-sitosterol glucoside, further confirmation was carried out by acid hydrolysis and identification of the sugar as glucose (PC, system VI) and the aglycone as β-sitosterol (m.p, mixed m.p and co-chromatography, TLC, system I).

Compound E-1: (Quercetin-3-O-β-D-glucoside)

It gave positive tests for carbohydrates and/or glycosides and flavonoids.[41] The UV spectral data with different ionizing and complexing reagents of compound E-1 (Table 5) with the physical behavior indicated that this compound is a flavonoid glycoside. ^{1}H-NMR showed the presence of five aromatic protons a pattern characteristic for quercetin and an anomeric sugar proton appear at δ 5.21 (1H, d, J= 7.6 Hz) indicating its β-nature, other sugar protons appear between δ 3.03-3.98. The UV and ^{1}H-NMR of compound **E-1** are similar to those reported for quercetin 3-O-β-D-glucoside.[48] Mild acid hydrolysis showed that compound **E-1** was hydrolyzed in one step indicated that it contain one sugar moiety. Complete acid hydrolysis gave aglycone identified as quercetin (m.p, mixed m.p, co-chromatography and UV spectral data) and one sugars identified as glucose (PC, system VI)

using authentic sugars. So compound E-1 was identified as quercetin-3-O-β-D-glucoside.

In conclusion: The results of this study showed that the total extract of *P. orientalis* and both of its chloroform and ethyl acetate fractions showed a blood glucose lowering effect on glucose loaded animals and anti-inflammatory effect on formalin-induced edema in mice indicating that *Pulicaria orientalis* can be used as an anti-diabetic agent for treatment of type II diabetes and also as an anti-inflammatory agent. Most of the isolated flavonoidal compounds either free or as glycosides were reported to exhibit such effects.[49-58] In addition, some compounds as β-sitosterol-O-β-D-glucoside and quercetin and its methoxylated derivatives showed an antitumour activity,[59,60] also β-sitosterol showed a strong anti-inflammatory activity.[61] This results together with the other reported results about the genus *Pulicaria* may support the folkloric uses of this genus. Further study is necessary for determination the LD_{50} and the accurate dose must be calculated.

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