Estrogenicity of Outer Scales of Onion on Reproductive functions of Mice

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ABSTRACT

Objectives: This study aimed to investigate the estrogen-like activities of the outer scales of onion and garlic. Methods: This work compared the estrogenic effects induced by estradiol and the plant extract in vivo and in vitro models of immature mice. Uterine wet weight/body weight ratios were determined. Uterotrophic bioassay (increased epithelial cell height, and gland development), immunohistochemical assay for the estrogen receptor and proliferative marker Ki67 and uterine contractility were investigated.

Results: Onion extract has the capacity to induce proliferative changes in the uterus that are analogous to those induced by estrogens, it also increased the wet uterine weight, and epithelial cell height. Also the frequency and amplitude of myometrial contractility were significantly increased in the group treated by onion extract. This estrogenic activity is attributed to quercetin and daidzein.

Conclusion: Our results support the possible estrogenic properties of the onion extract compounds.

INTRODUCTION

The importance of estrogens in homeostatic regulation of many cellular and biochemical events is well illustrated by the pathophysiological changes that occur with estrogen deficiency. As a result, natural estrogenic alternatives for the treatment of menopausal pathologies and symptoms are frequently considered, because this offers the hope of improved safety and greater compliance.

Onion and garlic worldwide-consumed vegetables are as a rich source of dietary quercetin known as strong anti-oxidant with ability to chelate metals and scavenge free radicals, which in turn inhibits lipid peroxidation. Many studies have presented other biological activities of quercetin that may be beneficial to health. It can inhibit platelet aggregation and/or broad spectrum of enzymes and has been demonstrated to have anti-inflammatory properties. Through these actions quercetin may contribute to protection from aging, vascular diseases and certain forms of cancer what suggests results of numerous epidemiological studies.[1]

Quercetin, a natural pentahydroxyflavone is believed to exhibit estrogenic and anticancer activity by acting as an effective radical-scavenger against oxidative cell damage. Various epidemiological studies have shown that quercetin and related isofla-

vonoids suppress cancerous tumor growth in vivo and in vitro.[2] Any structural and chemical similarities between the quercetin and synthetic estrogens can also provide insights to their mode of action.[3]

The present study tried to test the estrogenic effects of outer scales of onion and garlic in experimental animals hoping to find that they can be used for the replacement therapy and management of hormonal disturbance. Adjustment of doses was tried to avoid side effects when these plants are used in folk medicine, and in further studies, they can also be formulated in suitable pharmaceutical formulations. In the future, we plan to carry out pre-clinical and clinical trials for adopting pharmaceutical dosage forms with maximum efficacy and minimum side effects that can be prescribed to patients.

METHODS

This study was performed at the Department of Anatomy, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia through the years 2008-2009 after approval of the Ethical Committee of the Faculty.

A) Medicinal plants:

Red onion (A. cepa L.) and garlic (A. sativum) were purchased from local market. The outer red scales of the red onion and the white outer scales of garlic were air-dried, mechanically shredded then macerated in 50 % MeOH/ water. The filtered extracts were combined, concentrated under reduced pressure reconstituted in 100 ml MeOH.

B) Equipments:

In this study the following equipments were used: a Buchi rotavapour, Model R110; a. Freeze dryer Gamma 2-20, Martin Christ, Osterode, Germany. LKB Ultraspec UV-visible wave length spectrophotometer;

HPLC was carried out with a Shimadzu liquid chromatograph equipped with LC-10 AD pump, SPD-10A UV detector, Inertsil ODS-3 (200 mmx4.50 mmx5um) column for analytical purposes. An aliquot of 20 µL were injected. The flow rate of the mobile phase was 1ml/min.

Detection was carried out by a UV detector. Flavonoids were quantified at 360 nm, while phenolic compound were quantified at 280nm and the isoflavonoids were quantified at 254 nm.

C) Reagent

- Organic solvents: for the extraction procedures, e.g. ethanol, methanol, chloroform, ether, acetone and carbon tetrachloride, etc.
- HPLC grade water and acetonitrile (adjusted to PH 2 by phosphoric acid)
- Standard flavonoids: Rutin, hypersoid, quercetrin, quercetin, luteolin, narengenin, kampferol, sakurantin, chirisin and three hydroxyl flavon.
- Standard isoflavonoids: Trans-hydroxycinnamic acid, daidzein, trihydroxy isoflavon, genistein, isorhamnetin and formononetin.
- Standard phenolic compounds: Gallic acid, protocatechoic acid, pyrogallol, catechein, catechol, chlorogenic, caffeic acid, vanillic acid, caffeine, syrngic acid, ellagic acid, ferrulic acid, coumarin and cinnamic acid.

D) HPLC analysis of phenolic components:

Just before the HPLC analysis, a 400 _L aliquot of onion methanolic extract was evaporated near dryness. The residue was diluted back to 400 _L with the HPLC eluent (8 % CH3OH in HCOOH/H2O at pH=2.1) and filtered through 0.45_m PTFE filters (Millipore). The filtrate was analysed by means of high performance liquid chromatography (HPLC) using the equipment and chromatographic conditions, specified inTable1.

Detection was carried out for each sample subjected to HPLC analysis in three successive runs for phenolics (280nm) for isoflavonoids (254nm) and flavonoids (330nm).

Re-equilibration period was used between individual runs. The chromatograms were recorded at room temperature (20 °C).

E) Characterisation and quantification of phenolic compounds by HPLC:

Compounds were characterized on the basis of retention times and UV-VIS spectra of previously described standards and by using data of already published UV-VIS spectra [8–11, 13, 15, and 19].

Quantification of the major component was based on the peak areas of both standards and samples. Stock standard (diluted in methanol, 20-µg600 /ml) and samples were analyzed in duplicate.

F) Drugs:

1. 17 β -Ethynyl estradiol (E2) and peanut oil were obtained from Sigma Chemical Company (St. Louis, MO).
2. Estrogen receptor blocker (raloxifene) was obtained from Sigma Chemical Company (St. Louis, MO).

G) Animals:

Female wistar immature mice (aged 21 days) were kept under normal laboratory conditions, and given free access of food and water. They were left for acclimatization for 5days before starting the experiment.

H) Experimental Design

Animal Groups:

The mice were weighed and randomly divided into 6 groups, each consisting of 12 animals

Group I: control group. Rats didn't receive any medications throughout the experiment.

Group II: (estradiol treated group). Mice received daily subcutaneous injection of 1 mg / kg / day 17β estradiol in peanut oil for 7 days.[4]

Group III: (onion extract treated group). Mice received daily 30 mg /kg intraperitoneal injection of onion extract for 7 days.[5]

Group IV: Mice received daily intraperitoneal injection of 30 mg /kg onion extract preceded by subcutaneous injection of estrogen receptor blocker (raloxifene 5 mg/ Kg) for 7 days. [4]

Group V: (garlic extract treated group). Mice received daily intraperitoneal injection of 30 mg /kg garlic extract for 7 days.

Group VI: Mice received daily intraperitoneal injection of garlic extract preceded by subcutaneous injection of estrogen receptor blocker (raloxifene 5 mg/Kg) for 7 days.

Uterotrophic Assay

At the end of the study, the rats were weighed and blood samples were taken retro-orbitally for determination of estrogen level then animals were sacrificed by cervical dislocation. A full-length longitudinal abdominal incision was made to expose both uterine horns which were rapidly excised, the uteri were trimmed of fat and connective tissue and their wet weights were measured. Data were expressed as a percentage of the body weight.

Histological Study

One horn of each uterus was immediately fixed in buffered formalin 10%, then dehydrated in alcohol and embedded in paraffin. Three uteri from each of the experimental groups were taken for histological examination by haematoxylin-eosin staining. Five serial sections from the mid-horn of each uterine horn were compared.

Immunohistochemistry

Immunostaining for estrogen receptors and proliferative marker Ki 67 were performed on paraffin sections from the uteri of all groups. This were done using a primary anti serum to estrogen receptors and Ki 67(DAKO Corp. Denmark) followed by biotinylated horse antimouse antiserum, avidin-biotin complex and DAB as the chromogen. One of the uteri specimens was used as negative control by omitting the step of applying the primary antibody.

Morphometric studies:

Quantitation was performed with Leica image analysis computer system using the software Qwin 500 (England). It was used to study and compare the Luminal epithelial cell heights among the different groups used in this study.

Myometrial contractility:

4 longitudinal full-thickness myometrial strips, measuring 4.5 mm in length and 1.5 mm in width, were obtained from each horn.

The strips were mounted vertically, one end of the strip was connected to the lower hook

Figure 1: *Body weight, uterine wet weight (expressed as absolute values and as a percentage of body weight) of the different experimental groups.*

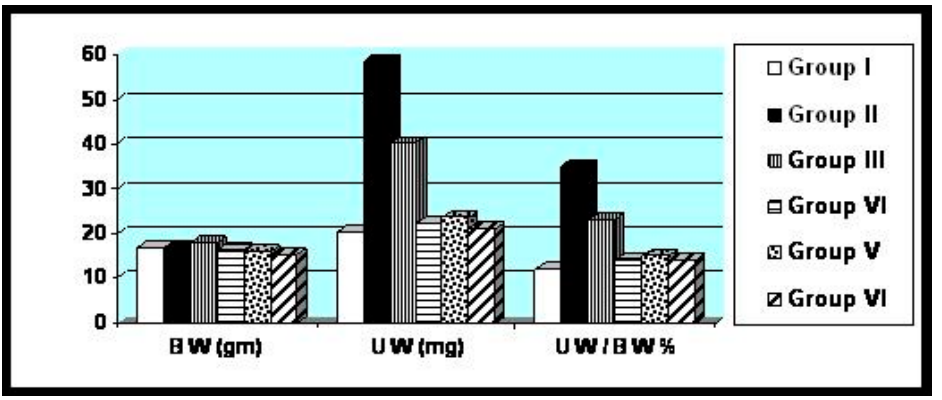
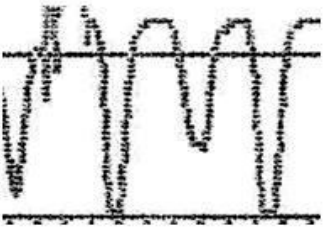


Figure 2: *Myometrial contractility in the control group*



Frequency (cycles/ min) and amplitude (screen units)

Figure 3: *Effect of 7 days treatment of onion extract 30 mg/Kg daily on myometrial contractility*

Frequency (cycles/ min) and amplitude (screen units)

Figure 4: *Photomicrographs showing the luminal epithelium(LE), Glandular epithelium(GE), lamina propria (LP), circular smooth muscle (CM) of the uterus from immature mice (A) Group I (B) Group II (C) Group III and (D) Group IV. Note the increase in the height of glandular epithelium in uteri exposed to E2 (B) and onion extract (C) more than that of control (A) and exposed to garlic extract (D). H&E; original magnification 400*

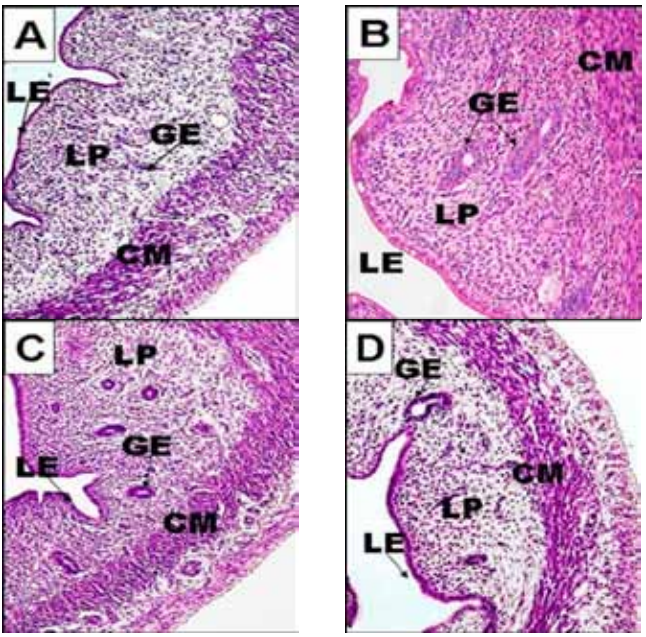
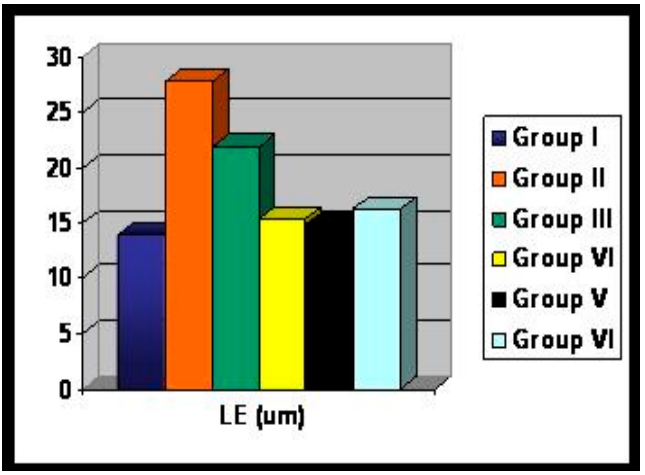


Figure 5: *Morphometric analysis of the height of luminal epithelium (LE) in the uteri of different experimental groups.*



of the bath and the other end of the strip was connected to a force transducer. The strips were incubated in the 20 ml tissue bath containing Krebs-Henseleit physiological solution (composition in mM: KCL 4.6; MgSO4 1.16; NaH2PO4 1.16; CaCl2 2.5; NaCl 115.5; NaHCO3 21.9; and glucose 11.1.), which were aerated continuously with 95% oxygen and 5% carbon dioxide. The pH was kept at 7.4 and the temperature was maintained at 37oC. The solution was constituted daily for each experiment. Myometrial strips were allowed to equilibrate for 20 min. The characteristics of the contraction including frequency and amplitude were recorded by an isometric force transducer of the intracel computerusing analyzing software Phys. 4 (Intracel Company England).

Statistical analysis of the results:

Results of the present study were presented as the mean ± SD. The significance of differences among different groups was determined by ANOVA test. Results were considered significant when probability P is <0.05.

RESULTS

Phytochemical results:

HPLC analysis of sample 1(the scales of red onion) showed the presence of major flavonoid was quercetin 18.8 % and the major isoflavonoid was daidzein 11.48%, while in sample 2 (the scales of red onion and garlic) the percentage of quercetin and daidzein were reduced 10%, 6.1% respectively and in sample 3 (the scales of garlic) trace amount of this active constituents whereas the phenolic compounds was predominate. Results are recorded in Table (1).

***Table 1**: Percentage of Phenolic constituents identified by HPLC in the methanolic extract of onion and garlic outer scales.*

Rt	Constituent	A	B	C
10.054	Quercetin	18.8	10	-
11.23	Kampherol	2.3	1.4	-
10.86	Narengenin	1.2	0.78	-
10.086	Luteolin	-	-	1.4
8.398	Quercetrin	-	-	-
3.303	Daidzein	11.48	6.1	-
4.066	Genistin	1.5	0.65	-
4.99	Isorhamnetin	1.2	0.45	-

Rt: retention time in minutes
A: onion sample
B: onion / garlic sample
C: garlic sample

Uterotrophic assay:

There was a significant increase in uterine wet weight in response to 30 mg/kg body weight onion extract and in response to E2 (positive control group) relative to the control group. The administration of garlic at concentrations of 30 mg/kg body weight had no effect on the wet weight of the uterus relative to the control group. Wet weight of the uterus, calculated as a percentage of the body weight, showed a similar pattern of change (Table 2, Figure 1).

Myometrial contractions:

Results are shown in table 3 and figures 2-3.

Spontaneous myometrial contractions were more frequent and having greater amplitude in onion extract treated group when compared to control group. Administration of estrogen receptor blocker together with the onion extract significantly decreased frequency and amplitude of contraction when compared to onion extract treated group I both the in vivo and in vitro experiments. Treatment with either estrogen or garlic extract did not significantly alter the isometric contractile parameters.

Uterine Morphology

The uterus exhibited a significant increase in luminal epithelial cell height, more development of the glandular epithelium and proliferation of the lamina propria in response to E2 and onion extract treatment relative to the control group (Figure 4, 5).

***Table 2**: Body weight, uterine wet weight (expressed as absolute values and as a percentage of body weight) of the different experimental groups:*

	Body Weight (gm)	Uterine Wet Weight (mg)	Uterine Wet Weight / Body Weight %
Group I	16.92 ± 0.34	20.12 ± 1.23	12%
Group II	16.83 ± 0.77	58.34 ± 1.30*	35%*
Group III	17.88 ± 0.95	40.33 ± 1.11*	23%*
Group VI	16.36 ± 1.20	22.10 ± 1.54	14%
Group V	15.84 ± 0.72	23.59 ± 1.70	15%
Group VI	15.23 ± 0.53	20.97 ± 1.69	14%

- Values for each group are expressed as mean ± SD. The control group (group I) represents pooled data from the control and the vehicle groups.
** Significantly different from the control group (p<0.05).*

***Table 3:** Frequency (cycles/ min) and amplitude (screen units) of contraction of uterine strips from all groups.*

	Group I	Group II	Group III	Group IV	Group V	Group VI
Frequency	0.64 ± 0.16	0.91 ± 0.18 P < 0.001 *	0.98 ± 0.18 P < 0.001 *	0.72 ± 0.13 P < 0.001 **	0.64± 0.13	0.5 ± 0.12
Amplitude	11.7± 1.7	14.7 ± 2.4 P < 0.005 *	15.3 ± 1.9 P < 0.001 *	12.9 ±2.2 P < 0.01 **	11 ± 1.6	11 ± 1.6

** Significant when compared with control group.*
** * Significant when compared with onion extract treated group.*

On the other hand, the other experimental groups were not different from the control group. Also, the myometrium within the uterus was not apparently affected in all the experimental groups.

Immunohistochemical Results

Within the luminal and glandular epithelium of the uterus, E2 and onion extract induced a prominent increase in the expression of ki 67 as aproliferator marker and also estrogen receptors (Figure 6, 7) relative to the control group. While the other groups showed weak immunoreactions.

Hormonal study:

Assessment of estrogen level in serum of all groups revealed insignificant difference except in the estrogen treated group which showed highly significant increase in serum estrogen level when compared to control group ($P < 0.001$).

COMMENT

To our knowledge, this is the first report to demonstrate that compounds isolated from onion scales show estrogenic activities in the female reproductive system. The present study clearly demonstrated the estrogenic responses to onion scales extract by uterotrophic, immunohistochemistry assays and in vivo and in vitro recording of myometrial contractility.

The implications of this study could be appreciated from both functional and therapeutic perspectives.

Reproductive tract function in the female is controlled primarily by the interaction of the ovarian sex steroids estradiol and progesterone. In the uterus, estradiol-17ß (E2) initiates a series of responses in uterine cells in preparation for the possibility of pregnancy, including cell hypertrophy and hyperplasia. Estradiole is also known to play a significant role in the regulation of myometrial contractility.[6] The actions of E2

Figure 6: *Uterine epithelial proliferation(Ki-67 immunostaining) A. in uteri of control mice, B. injected withE2 showing increase in epithelial proliferation, C uteri of mice injected with onion extract and D. uteri of mice injected with garlic extract. Original magnification x400*

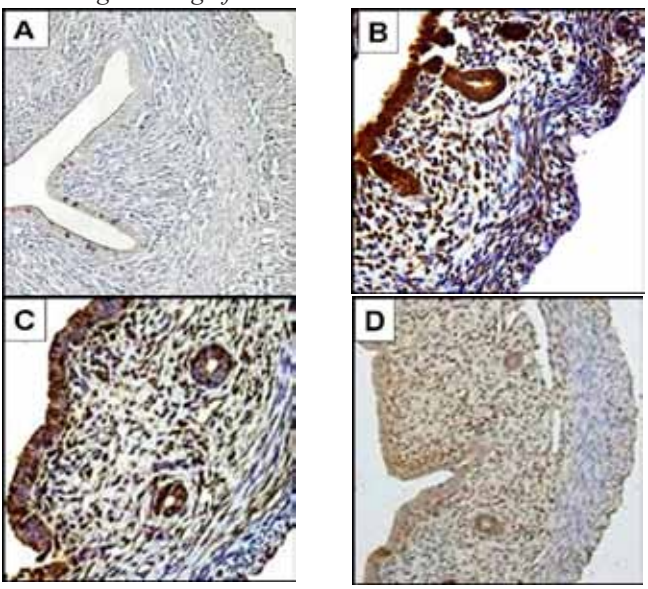
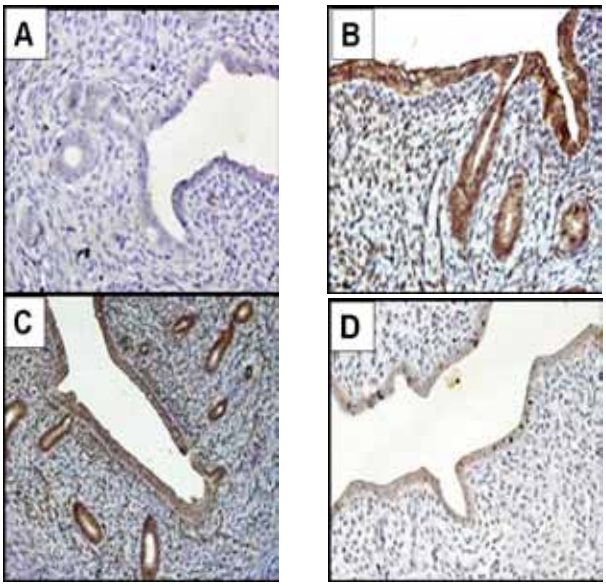


Figure 7: *Immunohistochemistry for Estrogen Receptors (ER) the uterus of. A) Group I uterine showed very weak staining, B) group II uterine section shows strong ER immunostaining in luminal and glandular epithelia and an ER-positive cell in the periluminal stroma . C) Group III immunostaining for ER is seen in epithelial cells, and ER-positive cells are detected in the stroma underlying the lumen and glands. D) Group IV ER immunostaining is faint in all epithelia and stroma. Original Magnifications x400.*



are mediated through binding specifically to estrogen receptors (ER), ligand-activated regulatory proteins that act as dimers on specific target genes containing defined DNA sequences called estrogen response elements (EREs). ER binding to EREs can result in induction or suppression of responsive genes. So, receptor's level influences target tissue responsiveness to E2.[7]

Circulating levels of estradiole and progesterone are low in anestrus rats or mice, but the uterus contains a full adult complement of receptors.[8] Thus one can inject anestrus animals with the appropriate hormone and study its effects on myometrial properties. So we injected anestrus mice with either 17 β estradiole, onion scales extract, garlic scales extract, or mixture of both extracts and we compared their effects on myometrial properties with each other and with control animals.

The current study revealed that the onion extract has the capacity to induce proliferative and stimulatory changes in estrogen target tissues (uterine endometrium and myometrium) that are analogous to those induced by estrogens. These effects have been identified in mice after exposure to onion extract concentrations of 30 mg /kg body weight. The ensuing morphologic changes include an increase in the wet weight, proliferation of luminal and glandular epithelium of the uteri and, and increase in epithelial cell height. Also the proliferative marker ki67 and estrogen receptors have been detected in significant amounts in the uteri of the onion extract group and also E2 group.

Our results also showed that onion scales extract treatment increased frequency and amplitude of contractions of uterine strips when compared to control none treated group.

Originally some investigators suggested that estrogen activates uterine contraction, and progesterone blocks it.[9,10]
In addition, Bek et al. (1988) showed that 1 µg 17 β estradiole injection to adult non pregnant rats is used to obtain spontaneous periodic contractions of myometrium.[11]

Many authors have investigated the possible mechanisms by which estrogen could enhance myometrial contractility. Early studies showed that estrogen stimulated oxytocin synthesis and release from the neurohypophysis and ovaries[12], in addition to well described estrogen stimulation of oxytocin receptors.[13]

Yallampalli and Dong (2000) stated that nitric oxide generation, which is known to inhibit myometrial contractility, by the uterus, was decreased in response to etradiole administration.[14] The same authors in another study (Dong and Yallampalli, 2000) observed that one dose of 5µg 17 β estradiole to ovarectomized rats significantly increased contractile prostaglandin F receptor mRNA expression in the uterus.[15]

Thota et al. 2003 observed that 5µg 17 β estradiole injection in ovarectomized rats for 3 consecutive days decreased the expression of mRNA of calcitonin receptor like receptor, CRLR and receptor activity modifying proteins, RAMP1,2, and 3 thus reducing the calcitonin gene related peptide and adrenomedullin induced inhibition of uterine contractility.[16]

An interesting finding in the present study is that injection of estrogen receptor blocker together with the onion scales extract attenuated the enhancement of myometrial contractility produced by injection of onion scales extract alone. In addition our immunohistochemistry staining results were reversed upon associating estrogen receptor blocker injection with the onion scales extract. These findings illustrate that active compounds in the extract (quercetin and daidzein) might produce their effects through binding to estrogen receptors. Furthermore the results of increased uterine weight strongly support these findings, as the uterine weight has been widely used as a sensitive parameter for evaluating estrogenic effect.[17]

The garlic scales extract did not show any significant changes in the uterotrophic, immunohistochemistry or uterine contractility assays whether the in vivo or the in

vitro experiments. This coincides with the phytochemical analysis which failed to show quercetin and daidzein compounds as that detected in the onion scales extract.

Our results support the hypothesis that onion scales extract contains specific estrogen like compounds (quercetin and daidzein). We are aiming to enhance our understanding of the spectrum of mechanisms that influence the biological responses of the uterus to these compounds and to achieve an appropriate formula that could be of value in the management of postmenopausal health disorders.

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