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EFFICACY OF LYOPHILIZED JUICE OF *CITRUS RETICULATA* FRUIT AS REPRODUCTIVE HEALTH SUPPLEMENT IN FEMALE RATS SUPPORTED BY PHYTOCHEMICAL ANALYSIS

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ABSTRACT
This study investigates the phytochemistry of lyophilized juice of *Citrus reticulata* fruit and evaluates its impact on the reproductive hormones of adult female rats. Phytoconstituents were identified by using phytochemical analysis, and GC-MS. The effect of lyophilized fruit juice on the oestrus cycle and reproductive hormones was assessed by using an in vivo experimental rat model. Phytochemical screening revealed the presence of amino acids, and fatty acids in the lyophilized fruit juice. The in vivo study demonstrated the estrogen antagonist effects of the lyophilized fruit juice in treated animals. This effect was attributed to the direct impact of the lyophilized fruit juice on female reproductive organs, leading to the suppression of follicular growth in the ovary or disruption of the hormonal balance in the hypothalamic-hypophysial ovarian and uterine axis. These findings highlighted the diverse array of phytochemical constituents present in the lyophilized juice of *Citrus reticulata* fruit and their potential effects on the reproductive hormones.

Keywords: GC-MS FAMES, Amino acid, Reproductive hormones, Citrus reticulata

INTRODUCTION
Maternal health, reproductive health, and fertility control are pivotal concerns in developing nations, where population growth outpaces the capacity of healthcare systems and the socioeconomic status of individuals. Certain bioactive compounds found in plants have been documented to negatively impact female reproductive organs and hormonal balance [1]. Understanding the potential pharmacological effects of these phytoconstituents could prove beneficial in addressing diverse female reproductive health needs. Plants containing bioactive compounds capable of modulating the body's hormonal profile may lead to hormonal imbalances and disruptions in fertility indices [2]. Advancements in the field of herbal medicine over the past decade have revolutionized treatment

modalities. The popularity of *Citrus reticulata*, which belongs to the family Rutaceae, has surged due to its herbal nature and therapeutic benefits. *Citrus reticulata* fruit exhibits significant antioxidant properties attributed to the presence of vitamins A and C, dietary fibers, proteins, and essential minerals (phosphorus, potassium, calcium, and magnesium). Additionally, they contain trace amounts of vitamins (B1, B2, B3, B5, B6, B9, and E)[3].

The fruit is also rich in amino acids, sugars, minerals, pectins, organic acids, and volatile compounds [4]. The antimicrobial efficiency of *Citrus reticulata* peel and fruit extracts is also reported in the literature [5]. However, research studies also emphasized on the nutritional richness and antioxidant potential of *Citrus reticulata* fruit, which can be utilized in therapeutic, culinary, and cosmetic applications [6].

Due to the copiousness of bioactive compounds, the current study aims to reconnoiter the phytoconstituents of *Citrus reticulata* fruit and evaluate their effects on the reproductive hormones of female rats.

MATERIAL AND METHODS

Plant material Collection and Authentication:

Citrus reticulata fruit was collected from the local market of Lahore. The fruits were authenticated by taxonomist Prof. Dr. Zaheer-ud-Din Khan, Department of Botany GCU Lahore. Plant material was preserved at the herbarium and Voucher No. GCU-1224 was issued.

Lyophilization of fruit juice

Fruit juice was extracted by cold press. Fruit juice was injected carefully into the vial for lyophilization. A small quantity of juice (3ml) was poured into vials for proper lyophilization with a micropipette. Sample vials were freezed at -40°C for 24 hours. For the primary and secondary phases of freeze drying, different temperature and pressure levels were adjusted according to set protocols [7].

Quantitative phytochemical analysis

Primary and secondary metabolites of the lyophilized fruit juice were estimated according to standard protocols i.e. total lipids, total carbohydrate [8, 9] total protein [10], total alkaloids, total flavonoids [11], total polyphenols [12].

Estimation of Amino Acid profile:

Amino acid analysis of lyophilized juice of *Citrus reticulata* fruit was executed on an amino acid analyzer (Biochrome 30+ Series) according to standard protocol 3CDEO (1998).

The process began by weighing 100mg of the lyophilized juice into sample bottles. Subsequently, 5 ml of performic acid solution was added to each bottle, and they were placed in the refrigerator for 16 hours. Next, 840mg of sodium disulfide was added to each sample bottle. Sample bottles were stirred for 10 minutes. After this gently 6 M HCl/Phenol solution was added with constant stirring. For one hour sample bottles were kept in the oven. Afterward, the samples were cooled, and placed in a box. The samples underwent another 24-hour incubation in the oven at 110°C. Once cooled, the samples were poured into a beaker while continuously stirring. The pH was maintained at 2.2 by adding 7.5M sodium hydroxide solution and the volume of samples was adjusted to 100 ml by adding distilled water. Subsequently, the samples were slowly injected into the sample tube of an amino acid analyzer.

Estimation of fatty acids by GC-MS (FAMES analysis)

For the evaluation of the fatty acid profile of lyophilized juice of *Citrus reticulata* fruit firstly, fat was extracted using diethyl ether, 50-60mg in a test tube, with a micropipette, then added 3ml 2, 2, 4 trimethyl pentane followed by addition of 0.5N sodium methoxide solution prepared in methanol was added, samples were mixed for 3min, then stayed for 15min. Sample (1µl) was injected into

GC-MS (7890-B). Samples were analyzed on a GC-MS 7890-B, Agilent Technologies. Extracted fat (0.5mg) was taken in a test tube, reacted with hydrogen chloride (methanol), and placed in the oven for 1hr. Extraction was performed by n-hexane, dried on sodium sulfate. 1µl was injected through a front auto liquid sampler and injected into GC-MS moreover; the temperature of the sample inlet and detector was also maintained at 200 and 250°C with a split ratio of 1:50. Quantification was done by FAME-37 standards [13].

Effect of lyophilized juice of *Citrus reticulata* fruit on reproductive hormones of adult female rats

For the current study, twenty adult female Wistar rats weighing between 150-200g were selected. These animals underwent a 2-week acclimatization period to ensure proper adaptation to their new environment and were weighed weekly thereafter. They were provided with unlimited access to food and water *ad libitum* throughout the study. The rats were divided into four groups, each consisting of six individuals: Group I (control), Group II (150mg/kg), Group III (250mg/kg), and Group IV (500mg/kg). The lyophilized juice was administered orally to the animals once a day using a straight ball-tipped needle for a duration of 28 days. Throughout the study period, the animals were monitored daily for behavioral activities, and their body weights were recorded daily. The estrous cycle was assessed by preparing vaginal smears of the animals, and the stages and duration of the cycle were determined according to the method outlined by Makonnen, Rostom [14]. To determine hormone levels, anesthesia was administered to the rats, and blood samples were collected via cardiac puncture. Subsequently, the blood samples were centrifuged to obtain serum, and the levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), estrogen, and progesterone were measured using the method detailed by Ahmed [15].

STATISTICAL ANALYSIS

All results are expressed as mean ± S.E. Data was analyzed by using one-way ANOVA followed by Dunnet multiple comparison test (Window SPSS 24). Results were considered significant at $P<0.05$.

RESULTS

Table 1: Estimation of different primary and secondary metabolites of lyophilized juice of *Citrus reticulata* fruit

Extract/ fraction	Total flavonoid content %	Total polyphenol Content %	Total Glycoside Contents %	Total Carbohydrate Contents %	Total protein Content %	Total lipids Content %
Lyophilized juice of *Citrus reticulata* fruit	21.2±0.4	20.7±0.3	1.30±0.3	25.2±0.1	15.2±0.1	8.2±0.1

Estimation of Amino Acid profile:

Amino acid profile analysis showed that there are ten types of amino acids present in the lyophilized juice of *Citrus reticulata* fruit. Out of the ten types of amino acids, seven were essential amino acids and three were non-essential amino acids. The concentration of these amino acids is shown in Table 2.

Table 2: Amino acid composition of lyophilized juice of *Citrus reticulata* fruit

Sr. No.	Name of amino acid	Percentage (%)
Essential amino acids(EAA) %		
1	Methionine	0.41
2	Threonine	0.38
3	Valine	0.52
4	Leucine	0.67

5	Isoleucine	0.61
6	Phenylalanine	0.69
7	Lysine	0.62
Non-essential amino acids(NEAA) %		
8	Cystine	0.13
9	Aspartic Acid	0.20
10	Serine	0.48

Estimation of fatty acids profile by using GC-MS (FAMES analysis)

FAMES analysis of lyophilized juice of *Citrus reticulata* fruit revealed the presence of 10 fatty acids as shown in the spectrum (Figure 3). The retention time and concentration of these fatty acids were presented in Table 3.

Table 3: Fatty acids profile of lyophilized juice of *Citrus reticulate* fruit

Sr. No	Name	RT	%age
01	Tetradecane	28.3	0.34
02	Lauric acid	31.2	3.57
03	Palmitic acid	32.2	0.91
04	9,12-Octadecadienal	33	0.309
05	Linoleic acid	33.8	49
06	Oleic acid	34.7	21.7
07	10-Octadecenoic acid	35.3	1.75
08	Myristic acid	37.1	2.92
09	Stearic acid	38.3	6.134
10	13,16-Octadecadiynoic acid	39.8	0.11

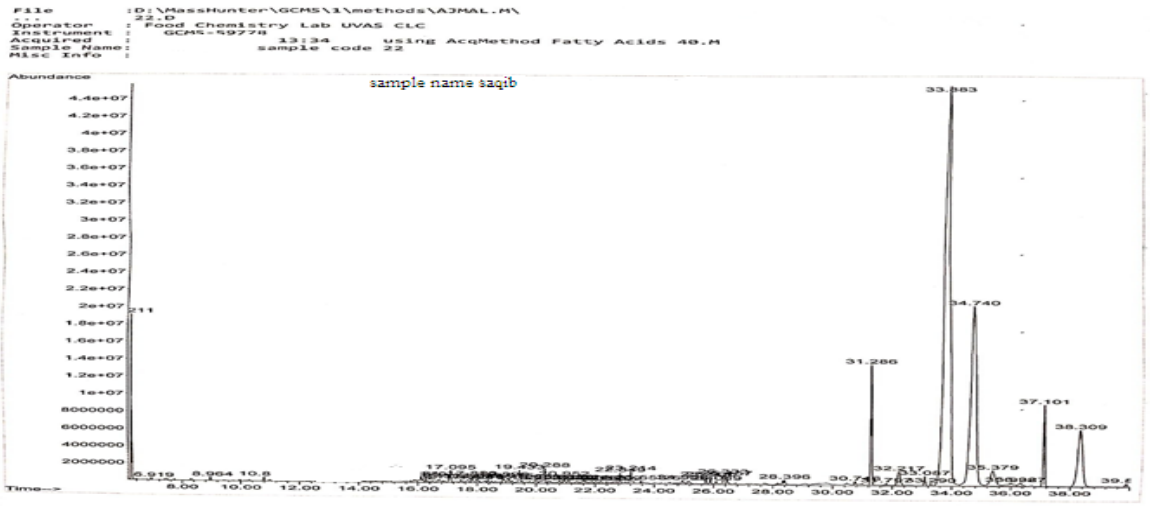

Figure 1: GCMS spectrum of fatty acids in lyophilized juice of *Citrus reticulata* fruit

Effect of lyophilized juice of *Citrus reticulata* fruit on reproductive hormones of adult female rats

The effects of the lyophilized juice of *Citrus reticulata* fruit are studied on the body and reproductive organs weight, the duration of the estrous cycle and its phases, and on reproductive hormone (LH, FSH, Estrogen, and Progesterone) levels in adult female rats. The lyophilized juice of *Citrus reticulata* fruit at doses of 150mg/kg (low), 250mg/kg (medium), and 500 mg/kg (high) orally. The results of these effects were shown in Table 4, 5, and 6.

Table 4: Effect of lyophilized juice of *Citrus reticulata* fruit on body and reproductive organs weight

Parameters	**Group I Control**	**Group II 150mg/kg**	**Group III 250mg/kg**	**Group IV 500mg/kg**
Body (g)	201.0±0.1	201.6±1.2	206.5±1.4*	218.6±0.2**
Ovary (mg)	32±0.3	30.2±1.1	31.6±1.3	38.6±0.1**
Oviduct (mg)	8.8±0.2	8.8±1.0	8.9±0.4	11.1±0.1**
Uterus (mg)	420.1±0.1	414.6±1.2	418.3±1.3*	440.4±0.3**
Vagina (mg)	150.1±0.0	150.6±1.3	152.6±1.1*	161.6±0.5**

n=6. Data represents mean±S.E; (P<0.05) * significant difference between the groups. (P<0.01) **significant difference as compared to the control group

Table 5: Effect of lyophilized juice of *Citrus reticulata* fruit on the duration of the estrous cycle and its phases

Phases of estrous cycle days	**Group I Control**	**Group II 150 mg/kg**	**Group III 250 mg/kg**	**Group IV 500 mg/kg**
Total duration	4.6±0.2	4.8±0.1	5.1±0.2	6.6±0.2**
Proestrus	1.2±0.2	1.6±0.1	1.6±0.2	1.8±0.2**
Estrous	1.6±0.4	1.6±0.3	1.6±0.3	1.8±0.1**
Metestrous	0.6±0.3	0.6±0.2	1.3±0.3*	1.7±0.1**
Diestrous	2.1±0.2	2.2±0.4	3.6±1.0*	4.6±0.3**

n=6. Data represents mean±SE; (P<0.05) * significant difference between the groups. (P<0.01) **significant difference as compared to the control group

Table 6: Effect of lyophilized juice of *Citrus reticulata* fruit on reproductive hormone levels

Hormones	**Group I Control**	**Group II 150 mg/kg**	**Group III 250 mg/kg**	**Group IV 500 mg/kg**
LH (mIU/mL)	0.9±0.3	0.6±0.4	0.13±0.2	0.03±0.2**
FSH (mIU/mL)	1.7±0.2	1.4±0.2	0.8±1.0*	0.1±0.3**
Estrogen (pg/mL)	145.6±0.1	145.1±0.3	136.2±0.4*	121.4±0.1**
Progesterone (pg/mL)	13.5±0.1	12.4±0.2	9.4±0.3*	4.1±0.1**

n=6. Data represent mean±SE; (P<0.05) * significant difference between the groups. (P<0.01) **significant difference as compared to the control group

DISCUSSION

In the present study phytochemical screening, GC-MS fames, and amino acid analysis were performed for standardization of lyophilized juice of *Citrus reticulata* fruit. Reports from literature prove that flavonoids are the compounds that are therapeutically active and responsible for a vast array of therapeutic outcomes on biological systems i.e., antioxidant, antimicrobial, anti-diabetic effects, anti-inflammatory effects, and antiarthritic effects of various plants [16]. Amino acids are the building blocks of protein present in animal and plant cells long protein chains are formed by peptide linkages of these amino acids. Amino acid sequencing in protein identification is important for the discovery of new drug molecules for the treatment of chronic metabolic disorders, cancer, and cardiovascular disease. Various amino acids were identified and quantified in the lyophilized juice of *Citrus reticulata* fruit as shown in Table 2. Research supported the therapeutic role of amino acids. Results of the analysis reported the presence of cystine which is anti-leukemia [17]. Essential amino acids, isoleucine, and leucine have the potential to control blood sugar levels, production of hormones, and wound healing. An insufficiency of these amino acids in the human body causes many health issues [18]. Leucine, decreased feed intake in animals was observed in animals with administration of isoleucine, and valine supplementation [19]. Lysine-rich diets play an important role in muscle development and protein synthesis [20]. Phenylalanine is the precursor of tyrosine

and part of food supplements Phenylalanine is present as a source of tyrosine. Glycine works as an antiacid, and analgesic, and is added as an ingredient in pet foods whereas, glutamic acid guards the brain from the venomous effect of chemicals [21]. A non-essential amino acid Aspartic acid, commercially used as low-calorie sweeteners and to develop biodegradable polymers [22] remained higher in concentration in lyophilized juice. Current study outcomes are in close covenant with the findings of Moro, Cabral [23] who reported the presence of above mention amino acid in plant extracts.

FAMES analysis revealed that the lyophilized juice of *Citrus reticulata* fruit is composed of several important fatty acids. Oleic acid, a major unsaturated fatty acid noted in the extract, is used as an excipient (pharmaceutical formulations), emollient and is recommended for use in managing hypercholesterolemia. Palmitoleic acid serves as a UV blocker, emollient, and promotes skin regeneration and skin protection. Other fatty acids identified include eicosadienoic acid, tridecanoic acid, tetracosanoic acid, arachidic acid, and behenic acid all of which have medicinal importance for treating various ailments. linolenic acid and Linoleic acid (49%) play a vital role in human health, particularly in treating hypercholesterolemia, cardiovascular diseases, and promoting reproductive health [24]. Human body can produce proinflammatory cytokines and adipokines when there are abnormal levels of myristic acid, behenic acid, and palmitic acid, which can initiate alarming conditions such as diabetes, obesity, cancer, and cardiovascular disease [25]. The cholesterol-raising potential of saturated fatty acids is determined by the length of the carbon chain [26]. The lyophilized fruit juice of *Citrus reticulata* consists of a small amount of these fatty acids, which are unlikely to be harmful to human health. Literature reports that palmitic acid has strong antibacterial and antifungal effects, while palmitoleic acid possesses potent antidiabetic, anti-inflammatory, and antiviral properties, and has beneficial effects on reproductive health [27].

In the current study, the oral administration of the lyophilized juice of *Citrus reticulata* fruit showed no significant change in body weight at all three dose levels after 28 days. However, genital organ weight was significantly increased at a 500mg/kg dose of lyophilized juice this might be due to the nutrient-rich composition of the juice. No signs and symptoms of toxicity and mortality appeared in rats with the administration of the lyophilized juice of *Citrus reticulata* fruit. This fact revealed the safety profile of the lyophilized juice of *Citrus reticulata* fruit at all used doses. Estrogen and progesterone are regulated by pituitary gonadotropins and regulate alterations in the female genital tract. Results showed reduced levels of FSH and LH which might be due to the effect of the lyophilized juice of *Citrus reticulata* fruit on the pituitary gland and hypothalamus. Gonadotropin-induced steroidogenesis also caused a significant decline in steroid hormones (estrogen and progesterone). Prolonged estrous cycle and diestrous phase were observed in treated animals which might be due to alleviated levels of estrogen.

The results of current research demonstrate estrogen antagonist effects of the lyophilized juice of In treated animals, *Citrus reticulata* fruit may mediate its effects through the direct action of lyophilized juice on female reproductive organs by disrupting the hormonal balance in the hypothalamo-hypophysial ovarian and uterine axis or by suppressing follicular growth in the ovary [15].

Furthermore, The isolation and characterization of active constituents from lyophilized juice of *Citrus reticulata* fruit through chromatographic and spectroscopic studies (NMR and mass spectroscopy) would lead to the identification of responsible phytoconstituent and its mechanism of action can bring revolutionary advancements in reproductive health medicine.

CONCLUSION

In conclusion, the present study reveals that the lyophilized juice of *Citrus reticulata* fruit contains diverse phytochemical constituents, including flavonoids, amino acids, and fatty acids. Furthermore, it demonstrates significant effects on the reproductive hormones of female rats. The isolation and characterization of active constituents from this fruit juice hold promise for the development of

standardized herbal formulations, potentially leading to revolutionary advancements in reproductive medicine.

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