doi.org/10.36721/PJPS.2022.35.2.SP.695-699.1

A new progestin from fungal transformation of norethisterone and its comparative antimicrobial studies

Muhammad Iqbal[1,2], Azizuddin[1]*, Syed Ghulam Musharraf[3], Saima Tauseef[4], Noreen Samad[5], Saima Khaliq[6] and Saara Ahmad[7]

[1]Department of Chemistry, Federal Urdu University of Arts, Science & Technology, Gulshan-e-Iqbal Campus, Karachi, Pakistan
[2]Government Degree Boys College, Jangal Shah, Keamari, Karachi, Pakistan
[3]H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Pakistan
[4]Department of Microbiology, Federal Urdu University of Arts, Science & Technology, Gulshan-e-Iqbal Campus, Karachi, Pakistan
[5]Department of Biochemistry, Bahauddin Zakariya University, Multan, Pakistan
[6]Department of Biochemistry, Federal Urdu University of Arts, Science & Technology, Gulshan-e-Iqbal Campus, Karachi, Pakistan
[7]Department of Biological and Biomedical Sciences, The Aga Khan University, Karachi, Pakistan

Abstract: Fungal transformation of a norethisterone (17α-ethynylestra-4-en-17β-ol-3-one) (**1**) by using *Macrophomina phaseolina* and *Paecilomyces variotii* was studied. A new metabolite, 17α-hydroxymethyl-androst-4-en-11β-ol-3-one-17β-acetate (**2**) with novel changes and a known metabolite, 17α-ethynylestradiol (**3**) were obtained from **1** by using *M. phaseolina* and *P. variotii*, respectively. Based on various spectroscopic techniques, the structures of both metabolites were characterized. The antimicrobial activities of **1**-**3** were also evaluated. Compound **1** was found to be moderately active against *Salmonella paratyphi* while **1**-**3** were almost inactive against other microorganisms.

Keywords: Fungal transformation, norethisterone, *Macrophomina phaseolina*, *Paecilomyces variotii*, antimicrobial activities.

INTRODUCTION

The remarkable regio- and stereoselective fungal transformation of steroidal compounds has been used for several decades to acheive more beneficial steroidal drugs at environmental friendly conditions. The process is used to detect new microorganisms and reactions. Filamentous fungi are rich in a variety of enzymes, which are responsible for various reactions such as hydroxylation, acetylation, oxidation, reduction, double bond formation, aromatization etc. in order to get structurally diverse form of substrate (Sultana, 2018; Azizuddin *et al*., 2020; Wang *et al*., 2021).

Synthetic progestogens, having identical function to progesterone are called progestins. In 1939, for the first time they were brought in for medical use but in 1950s, they were launched to be used in family planning (Kuhl, 2011). Norethisterone (NET) (**1**) is a nortestosterone derivative, an effective synthetic steroidal sex hormone having very poor estrogenic and androgenic properties. It was manufactured as well as patented by Syntex Company, Mexico City. It is used to postpone menses, avoid unintended pregnancies and treat endometriosis. Comparative studies of progestins concluded that it is the safest among all female contraceptives (Taniguchi *et al*., 2017).

In the present work, we have described fungal transformation of NET (**1**) using *M. phaseolina* and *P. variotii* for the first time along with antimicrobial activities of **1**-**3** against several microorganisms (fungi and bacteria).

**Corresponding author:* e-mail: azizpobox1@yahoo.com

MATERIALS AND METHODS

General experimental conditions

Norethisterone (**1**) was isolated from a well-known sample drug. The melting point was determined in a glass capillary tube using ST15 OSA, UK melting point apparatus whereas optical rotation was determined on a polarimeter (JASCO P-2000) using $CHCl_3$ as a solvent. Silica gel (60-270 mesh) column was used for the separation of transformed products. To examine the purity of samples, pre-coated silica gel TLC plates (20×20 cm, 0.25 mm thick, Merck) were used. The LREI-MS and HREI-MS were recorded on Finnigan MAT 311 and Jeol JMS 600 mass spectrometers, respectively. JASCO A-302 spectrophotometer was used for IR spectroscopy while Bruker Avance-400 NMR spectrometer was used to get ^{1}H- and ^{13}C-NMR spectra.

Fungal transformation

The fungal cultures *Macrophomina phaseolina* (ATCC 64334) and *Paecilomyces variotii* (ATCC 1104) were used in the experimental work. These fungal cultures were grown and stored on Sabouraud dextrose agar (SDA) slant at 4ºC. Liquid nutrient broth for each fungus was made by dissolving the given chemicals: glycerol (15 mL), yeast extract (15 g), peptone (15 g), glucose (30 g), KH_2PO_4 (15 g) and NaCl (15 g) into distilled water (3 L). The nutrient broth was distributed in 50 flasks of 250 mL (60 mL in each flask), and autoclaved at 121ºC for 1 hour.

Amongst the 50 flasks, two were used as control in the experiment. One of them (contain media and substrate) was used to examine the stability of the substrate whereas the other (contain media and fungus) was used to check the metabolites of fungus. Fungal spores were inoculated into 48 flasks and placed on a shaker. After two days, the saturated solution of **1** (300 mg in 16.5 mL acetone) was distributed equally among 48 flasks, containing mature fungal growth and placed on a shaker. The reaction in the flask was stopped after every two days to check the transformation of **1**. The biomass was filtered and extracted with thrice volume of CH_2Cl_2. The solvent from the extract was evaporated. Control flasks were also extracted in a similar way. A comparative TLC was carried out, which showed the bioconversion of **1**. The gummy extracts 0.28 g (from *M. phaseolina*) and 0.46 g (from *P. variotii*) were adsorbed on flesh silica gel for column chromatography. Elution with ethyl acetate:pet.ether (25:75) yielded **2** (13.4%) while ethyl acetate:pet.ether (12:88) solvent system yielded **3** (48.2%) from the crude extracts of *M. phaseolina* and *P. variotii*, respectively.

Antimicrobial activities procedure

Agar well method was used to evaluate antimicrobial activities (Bauer *et al*., 1966). The fungal cultures were grown on SDA slant, and stored at 4ºC until required for use. Water was distilled and autoclaved. Fungal spores' suspension was prepared into autoclaved distilled water and transferred identically into all SDA plates. The incubation of all plates was carried on for 24-48 hours at 28±2 ºC. Thereafter fungal growth was observed by measuring the diameter of area of inhibition. Distilled water was used as negative control whereas griseofulvin (antifungal agent) as a positive control. Griseofulvin had > 20 mm zone of inhibition.

The bacterial cultures were cultivated on nutrient agar (NA) slant, and stored at 4ºC. Iso-sensitized agar was used to prepare plates. The stock solution for each progestin was prepared in DMSO. Germ-free discs that contain 10µL solution of corresponding compound were utilized for screening. The autoclaved discs were seeded with 24 hours old cultures of bacteria. The prepared discs were placed on agar surface at various points and the plates were nourished at 37ºC for 24 hours. The outcomes were verified by measuring the zone of inhibitions in mm. DMSO was employed as negative control whereas antibiotics amoxicillin and ampicillin were utilized as positive control.

STATISTICAL ANALYSIS

Samples were analyzed in three replicates for antimicrobial activities. Results were expressed as mean ± S.E.M.

RESULTS

Microbial transformation of NET (**1**) with *M. phaseolina* and *P. variotii* afforded **2** and **3**, respectively (fig. 1).

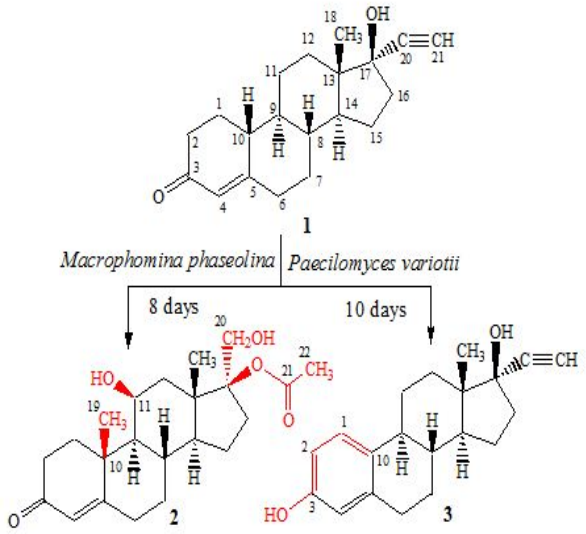

Fig. 1: Fungal transformation of norethisterone (**1**) with *M. phaseolina and P. variotii*.

Norethisterone (1)

White to creamy crystalline solid; m.p.: 201-203ºC; $[\alpha]_D^{25}$: -30.6º (*c* 0.1, $CHCl_3$); IR ($CHCl_3$) ν_{max}: 3400 (OH), 3300 (≡C-H), 2130 (C≡C), 1650 (C=O), 1590 (C=C) cm^{-1}; LREI-MS *m/z*: 298 $[M]^+$; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 5.80 (1H, s, H-4); 2.54 (1H, s, H-21), β 2.37, α 2.24 (2H, m, H-2), β 2.06 (1H, ddd, *J* = 8.4, 8.4 and 4.6 Hz, H-10), β 2.23, α 1.52 (2H, m, H-1), α 1.89, β 1.22 (2H, m, H-11); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 199.3 (C-3), 87.2 (C-20), 79.6 (C-17), 74.0 (C-21), 42.5 (C-10), 36.4 (C-2), 26.5 (C-1), 26.1 (C-11).

17α-Hydroxymethyl-androst-4-en-11β-ol-3-one-17β-acetate (2)

Colorless crystalline solid; m.p.: 170-172ºC; $[\alpha]_D^{25}$: +118º (*c* 0.1, $CHCl_3$); IR ($CHCl_3$) ν_{max}: 3412 (OH), 1710 (C=O), 1655 (C=O), 1575 (C=C) cm^{-1}; LREI-MS *m/z*: 376 $[M]^+$; ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz) complete data in table 1.

17α-Ethynylestradiol (3)

White solid; m.p.: 180-182ºC; $[\alpha]_D^{25}$: -132º (*c* 0.1, $CHCl_3$); IR ($CHCl_3$) ν_{max}: 3304 (≡C-H), 3295 (OH), 2130 (C≡C), 1610, 1515 (C=C) cm^{-1}; LREI-MS *m/z*: 296 $[M]^+$; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 7.04 (1H, d, *J* = 8.3 Hz, H-1), 6.58 (1H, dd, *J* = 8.3 and 2.8 Hz, H-2), 6.48 (1H, d, *J*=2.8 Hz, H-4); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 154.3 (C-3), 132.4 (C-10), 127.2 (C-1), 114.8 (C-4), 112.4 (C-2).

Antimicrobial activities

The results of antimicrobial activities including antifungal and antibacterial activities are listed in tables 2 and 3, respectively.

Table 1: ^{13}C-NMR[a])[b]) and ^{1}H-NMR[c]) spectral data of new compound **2**.

S No.	δ_C	δ_H (*J* in Hz)
1	33.8 (CH_2)	α 1.12 (m), β 2.06 (m)
2	32.7 (CH_2)	α 1.99 (m), β 2.07 (m)
3	199.5 (C)	-
4	122.1(CH)	5.66 (s)
5	170.6 (C)	-
6	35.0 (CH_2)	α 2.50 (m), β 2.36 (m)
7	32.0 (CH_2)	α 2.03 (m), β 1.98 (m)
8	31.4 (CH)	β 2.05 (m)
9	55.4 (CH)	α 1.00 (m)
10	39.2 (C)	-
11	68.7 (CH)	4.45 (ddd, *J* = 3.5, 3.0, 3.0)
12	34.5 (CH_2)	α 2.76 (m), β 2.17 (m)
13	47.6 (C)	-
14	52.0 (CH)	α 1.68 (m)
15	23.6 (CH_2)	α 1.81 (m), β 1.44 (m)
16	39.8 (CH_2)	α 2.05 (m), β 1.72 (m)
17	89.6 (C)	-
18	17.1 (CH_3)	0.95 (s)
19	21.0 (CH_3)	1.42 (s)
20	67.8 (CH_2)	4.80 (d, *J*=17.5), 5.02 (d, *J*=17.5)
21	204.7 (C)	-
22	20.5 (CH_3)	2.16 (s)

[a]) Multiplicities were determined by DEPT experiments.
[b]) Assignment based on HMQC and HMBC.
[c]) Assignment based on COSY and HMQC.

DISCUSSION

The enzymatic reactions of fungi are special in their significance and diversity. The culture of fungus contains enormous specific enzymes that provide access to unreachable sites in steroidal compounds. In this research paper, we explored that the fungus *Macrophomina phaseolina* has some novel changes including hydroxylation, methylation, acetylation and reductive-elimination in **1**, and yielded a new compound **2**, whereas the enzymatic machinery of fungus *Paecilomyces variotii* has ability to bring the ring-A aromatization of 19-nor steroid **1** to yield a known compound **3** in a short period of time at green conditions.

The LREI-MS of **2** showed M^+ at *m/z* 376, which was 78 units more than substrate **1**, indicating the substitution of small atom or group of atoms by a large group of atoms. The HREI-MS of **2** showed the M^+ at *m/z* 376.1354, corresponding to $C_{22}H_{32}O_5$, (calcd. 376.1352). The IR spectrum ($CHCl_3$) of **2** was quite different from **1** due to the absence of acetylenic absorption and presence of an extra band at 1710 (C=O) cm^{-1} indicating changes at C-17 position. The IR spectrum of **2** also displayed characteristic absorptions at 3412 (OH), 1655 (C=O) and 1575 (C=C) cm^{-1}.

The ^{1}H-NMR spectrum of **2** (table 1) was noticeably different from **1** having an extra downfield methine signal at δ_H 4.45 (ddd, *J* = 3.5, 3.0 and 3.0 Hz, H-11) indicating the presence of -OH group in **2**. The position of -OH was suggested at C-11 by the disappearance of multiplet signals for C-11 protons at δ_H β 1.22 and δ_H α 1.89 in **2** as compared to **1**. In the ^{1}H-NMR of **2**, the disappearance of acetylenic proton at δ_H 2.54 (s, H-21), and appearance of downfield methylene signals at δ_H 4.80 (d, *J* =17.5 Hz, H-20) and δ_H 5.02 (d, *J* =17.5 Hz, H-20) as compared to **1**, showed the conversion of acetylenic group into hydroxymethyl (-CH_2-OH). The ^{1}H-NMR of **2** also displayed downfield singlet for a methyl at δ_H 2.16 (s, H-22), which was absent in **1**, indicating the *O*-acetylation at C-17. Furthermore, the disappearance of C-10 methine proton at δ_H β 2.06 (ddd, *J* =8.4, 8.4 and 4.6 Hz) and appearance of a methyl at 1.42 (s, H-19) as compared to **1**, indicated the methylation in **2**. The rest of the ^{1}H-NMR of **2** was almost similar to **1**.

The ^{13}C-NMR spectra of **2** (table 1) showed four additional downfield signals at δ_C 204.7 (C), δ_C 89.6 (C), δ_C 68.7 (CH) and δ_C 67.8 (CH_2) along with an upfield quaternary carbon at δ_C 39.2 as compared to **1**. The ^{13}C-NMR spectra of **2** also displayed two extra methyl signals at δ_C 20.5 and δ_C 21.0, which were absent in **1**. Furthermore, the absence of acetylenic quaternary carbon in **2** and presence of a downfield methylene signal at δ_C 67.8 (C-20) as compared to **1**, indicated the conversion of acetylenic group into hydroxymethyl (-CH_2-OH). The presence of additional signals of a carbonyl and a methyl at δ_C 204.7 (C-21) and δ_C 20. 5 (C-22), respectively indicated the *O*-acetylation at C-17. The downfield shifted C-17 quaternary carbon at δ_C 89.6 also suggested the above changes. The ^{13}C-NMR spectra of **2** also displayed the shifting of upfield methylene carbon at δ_C 26.1 (C-11) of **1** into a downfield methine at δ_C 68.7 in **2** along with downfield shifted signals for C-12 methylene at δ_C 34.5 and C-9 methine at δ_C 55.4, which were present at δ_C 32.3 (C-12) and δ_C 49.0 (C-9) in **1**, suggesting the presence of hydroxyl group at C-11 position. The disappearance of C-10 methine at δ_C 42.5 and appearance of upfield C-10 quaternary carbon signal at δ_C 39.2 along with a methyl at δ_C 21.0 (C-19), indicated the methylation at C-10 in **2**.

The HMBC spectrum of **2** showed correlations between methylene protons of C-12 (δ_H α 2.76 and δ_H β 2.17) and methine proton of C-9 (δ_H α 1.00) with C-11 (δ_C 68.7). In the HMBC spectrum, downfield shifted methine proton of

Table 2: Antifungal activity of NET (**1**) and its metabolites **2** and **3**.

S. No.	Name of fungi	Zone of inhibition (mm)		
		1	**2**	**3**
1	*Candida albicans*	-	-	-
2	*Candida tropicalis*	03±1	-	-
3	*Candida krusei*	06±1	04±1	-
4	*Saccharomyces cerevisiae*	07±1	06±1	07±1
5	*Aspergillus niger*	03±1	03±1	07±1
6	*Aspergillus flavus*	-	-	04±1
7	*Rhizopous* sp.	-	-	-

Table 3: Antibacterial activity of NET (**1**) and its metabolites **2** and **3**.

S. No.	Name of bacteria	Zone of inhibition (mm)		
		1	**2**	**3**
1	*Streptococcus pneumonia*	-	-	-
2	*Staphylococcus aureus*	-	-	-
3	*Salmonella typhi*	07±1	05±1	-
4	*Salmonella paratyphi*	13±1	06±1	09±1
5	*Haemophilus influenza*	10±1	-	-
6	*Klebsiella pneumonia*	-	-	-
7	*Escherichia coli*	-	-	-
8	*Pseudomonas aerogenosa*	-	-	-
9	*Pseudomonas*	-	-	-
10	*Entrobacter*	11±1	09±1	07±1
11	*Proteus*	10±2	-	-

C-11 (δ_H α 4.45) showed ^2J correlations with C-9 (δ_C 55.4) and C-12 (δ_C 34.5) specifying C-11 hydroxylation. The C-9 methine proton (δ_H α 1.00) exhibited ^2J correlation with C-10 (δ_C 39.2) supporting methylation at C-10 position whereas the C-20 methylene protons (δ_H 4.80 and δ_H 5.02) showed correlations with C-17 (δ_C 89.6) and C-21 (δ_C 204.7) indicating the absence of acetylenic group. The protons of C-22 methyl (δ_H 2.16) showed correlations with C-21 (δ_C 204.7), also confirming *O*-acetylation of hydroxyl group at C-17 (fig. 2).

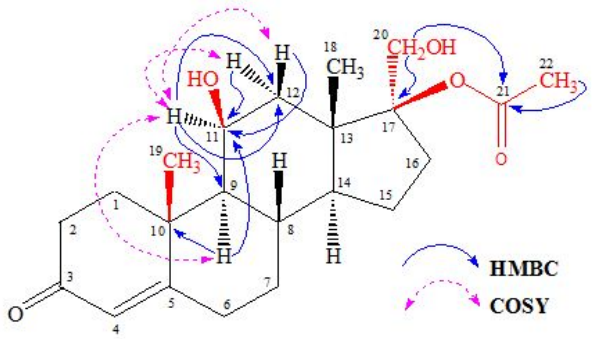

Fig. 2: Key HMBC and COSY interactions in **2**.

The COSY-45º of **2** showed the vicinal coupling of C-11 methine proton (δ_H 4.45) with the methylene protons of C-12 (δ_H β 2.17 and α 2.76) and C-9 methine proton (δ_H 1.00) further specifying the presence of -OH at C-11 (fig. 2). The NOESY spectrum of **2** displayed interaction of α-oriented H-14 (δ_H 1.68) with H-11 (δ_H 4.45) and H-9 (δ_H 1.00), assigning the β-configuration of geminal -OH group at C-11 (fig. 3).

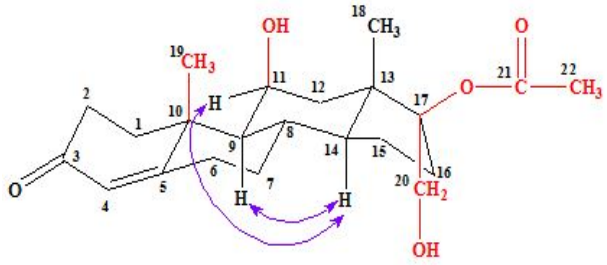

Fig. 3: Key NOESY correlations in **2**.

Based on the above available spectral data of **2**, it is proposed that it was a new compound, 11β-hydroxy-17α-hydroxymethyl testosterone acetate (**2**). We reported for the first time **2** from **1** by using *Macrophomina phaseolina* with interesting and novel changes. The literature showed that microorganisms, animals and human could not cause the methylation of **1** but the deethynylation was observed in human (Azizuddin and Iqbal, 2020). In addition to these two novel changes, the fungus *M. phaseolina* hydroxylated **1** at C-11 position, which was well documented in the literature for almost all fungi (Mahato and Mukherjee, 1984) as well as acetylated 17β-hydroxyl group, which was also common for fungi (Faramarzi *et al*., 2002; Choudhary *et al*., 2011).

The parallel spectroscopic study of **3** with the published data showed that it was a known compound, 17-α-ethynylestradiol (França *et al*., 2017). We reported for the first time **3** from **1** by using a new microbial source, *Paecilomyces variotii.*

Antimicrobial activities

The antifungal activity of **1**-**3** was checked against four yeasts and three saprophytic fungi but all compounds were almost inactive against all fungi. On the other hand **1**-**3** were inactive against *Klebsiella pneumoniae*, *Streptococcus pneumonia*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aerogenosa* and *Pseudomonas* whereas only substrate **1** was found to be moderately active against *Salmonella paratyphi* (13±1).

CONCLUSION

In this research work, we reported the fungal transformation of NET (**1**) by using new fungal strains and antimicrobial activities of **1**-**3**. We explored novel changes in **1** by using *M. phaseolina* as well as ring-A aromatization of 19-nor steroid by fungus *P. variotii*. The antimicrobial results showed that compounds **1**-**3** were almost inactive against most of the microorganisms. Compound **1** demonstrated better activity against *Salmonella paratyphi.* The results would be useful to synthesize more diverse form of NET (**1**) in green conditions.

REFERENCES

Azizuddin and Iqbal M (2020). A review on the biotransformation of synthetic progestin, norethisterone. *Pak. J. Biotechnol.*, **17**(4): 195-203.

Azizuddin and Iqbal M, Musharraf SG and Shahzad S (2020). Stereoselective microbial hydroxylation of progestin, norethisterone by using *Aspergillus niger* and *Penicillium citrinum*. *Nat. Prod. Sci.*, **26**(4): 283-288.

Bauer AW, Kirby WMM, Sherris JC and Turck M (1966). Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Path.*, **45**(4): 493-496.

Choudhary MI, Erum S, Atif M, Malik R, Khan NT and Atta-ur-Rahman (2011). Biotransformation of (20*S*)-20-hydroxymethylpregna-1,4-diene-3-one by four filamentous fungi. *Steroids*, **76**(12): 1288-1296.

Faramarzi MA, Yazdi MT, Shafiee A and Zarrini G (2002). Microbial transformation of hydrocortisone by *Acremonium strictum* PTCC 528. *Steroids*, **67**(10): 869-872.

França JAA, Navarro-Vázquez A, Lei X, Sun H, Griesinger C and Hallwass F (2017). Complete NMR assignment and conformational analysis of 17-α-ethinylestradiol by using RDCs obtained in grafted graphene oxide. *Magn. Reson. Chem.* **55**(4): 297-303.

Kuhl H (2011). Pharmacology of progestogens. *J. Reproduktionsmed. Endokrinol.*, **8**(1): 157-177.

Mahato SB and Mukherjee A (1984). Steroid transformations by microorganisms. *Phytochemistry*, **23**(10): 2131-2154.

Sultana N (2018). Microbial biotransformation of bioactive and clinically useful steroids and some salient features of steroids and biotransformation. *Steroids*, **136**: 76-92.

Taniguchi F, Enatsu A, Ikebuchi A, Yamane E, Moriyama M, Murakami J, Harada T and Harada T (2017). Efficacy of norethisterone in patients with ovarian endrometrioma. *Yonago Acta Med.*, **60**(3): 182-185.

Wang F, Chen L, Chen S, Chen H and Liu Y (2021). Microbial biotransformation of pericarpium citri reticulatae (PCR) by *Aspergillus niger* and effects on antioxidant activity. *Food Sci. Nutr.*, **9**(2): 855-865.