

Scientific Foundation SPIROSKI, Skopje, Republic of Macedonia  
 Open Access Macedonian Journal of Medical Sciences. 2020 May 25; 8(A):434-440.  
<https://doi.org/10.3889/oamjms.2020.4418>  
 eISSN: 1857-9655  
 Category: A - Basic Sciences  
 Section: Pharmacology



# Cyclea barbata Miers Ethanol Extract and Coclaurine Induce Estrogen Receptor $\alpha$ in the Development of Follicle Pre-ovulation

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## Abstract

**BACKGROUND:** Menstrual disorders to women are generally caused by the disturbance of estrogen production level. This can cause problems in the female reproductive organs. Herbal medications at this moment are demanding to prevent the risk of chemical substances, one of them is grass jelly leaves (*Cyclea barbata*) which has the potency to induce estrogen levels to women and some people use it to overcome fertility problems. One of the active compounds content is coclaurine.

**AIM:** The study aimed to know the main compound leaves of *C. barbata* which has an important role in estrogen induction, protein target, and pathway through to activate estrogen production.

**METHODS:** There are 18 active compounds from *C. barbata* gained from database and had been confirmed the presence of coclaurine compound used liquid chromatography-mass spectrometry. Then, it predicted that the potency of molecular interaction between active compounds of ESR $\alpha$  had been done by the molecular docking and *in vitro* approach.

**RESULTS:** Coclaurine has the highest binding affinity to activate ESR $\alpha$ . Even though the network analysis prediction did not show the direct interaction, we predict the coclaurine might first interact with other steroid proteins showing by Cytoscape analysis. *In vitro* results showing the maximum dosage of *C. barbata* extract is in 100 ppm, while coclaurine dosage is optimum at 100 and 200  $\mu$ M/L to induce the maturation of oocytes.

**CONCLUSION:** Coclaurine compounds give stimulation of hormonal induction through several proteins such as SRC, ADRB2, and ADRB3 which involve CYP1A1 and CYP19A1, then activate estrone and estradiol to help the increase of estrogen. *C. barbata* ethanol extracts and coclaurine had been proved having ability to enhance development follicles pre-ovulation.

**Edited by:** Igor Spiroski  
**Citation:** Noviyanti N, Yueniwati Y, Ali M, Rahardjo B, Permatasari GW. *Cyclea barbata* Miers Ethanol Extract and Coclaurine Induce Estrogen Receptor  $\alpha$  in the Development of Follicle Pre-ovulation. Open Access Maced J Med Sci. 2020 May 25; 8(A):434-440. <https://doi.org/10.3889/oamjms.2020.4418>  
**Keywords:** Coclaurine; *Cyclea barbata*; ESR $\alpha$ ; Molecular docking; Follicle pre-ovulation  
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**Received:** 18-Feb-2020  
**Revised:** 05-Apr-2020  
**Accepted:** 14-Apr-2020  
**Copyright:** © 2020 Noviyanti Noviyanti, Yuyun Yueniwati, Mulyohadi Ali, Bambang Rahardjo, Galuh W Permatasari  
**Funding:** This research did not receive any financial support  
**Competing Interests:** The authors have declared that no competing interests exist  
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## Introduction

Menstruation cycles are one of the indicators in the women fertility. Reproduction problems to women cover the disturbance in the development of ovum, tube, and uterus due to hormonal disturbance, reproduction organ growth, nutritional status, stress, age, and metabolic disease such as diabetes mellitus. These cases impact to the occurring of menstruation cycles [1], [2]. The cases of reproduction disturbance were as many as 9% cases experienced by husband/wife spouse in the world [3].

The occurring of menstruation relates to the ovarian function that is releasing steroid hormone such as estrogen and progesterone. The low level of estrogen inside the body impacts to the period disturbance/ menstruation cycles. Interaction between granulosa layer and theca layer will result in the increase of estrogen production. The lack of gene expression activity CYP19 aromatase caused a decrease of estrogen. Aromatase

is cytochrome enzyme P450 which catalyzed conversion of androstenedione and testosterone into estrone and estradiol [4], [5]. The giving of estrogen helps to stimulate the growth maturity of follicle, oocyte development, and embryo production [6].

The medication of ovulation disturbance now is directing to the non-chemical therapy or called herbal therapy [7]. One of the plants use to heal menstruation disturbance is grass jelly leaves (*Cyclea barbata*) [8].

There is a high antioxidant compound in the leaves *C. barbata* extracts those are alkaloid, flavonoid, terpenoid, tannin, glucosidal, saponin, and antrakuinon [7]. Based on the informatics study, these sevens compounds, there is a compound type which has a chemical structure resemble to estradiol chemical structure 17- $\beta$  that is a steroid. Steroid may be come from triterpenoid and also to alkaloid (coclaurine). Many alkaloid has terpenoid character to plant such as solanine, alkaloid steroid potato, and solanum,

thus called terpenoid modification [9]. These active substances have positive effect as the fertility controller medication [9], [10].

Based on the database information in PubChem, there are 18 active compounds in the leaves of *C. barbata*, one of them is coclaurine which has steroid structure and strong bond interaction with receptor estrogen  $\alpha$ . This research focused to the exploration of *C. barbata* extract and coclaurine compound potency molecularly to women fertility, particularly to the estrogen hormone used *in silico* and *in vitro* approach.

## Materials and Methods

### Extraction leaves samples of *C. barbata*

The samples of grass jelly leaves (*C. barbata*) come from Manna Regency of South Bengkulu. The leaves were taken in the morning under running water and weighing with weight was 437,558 g. Then, the leaves put into the oven with a temperature 70°C till dried. The oven result in gained weight was 437,303 g. The dried leaves grind with a girder of screening measurement 0.2 mess. The weight of leaves samples of grinding grinder result was 369,28 g. Then, submerged in the pure ethanol solution, shaken for 30 min, and submerged for 24 h (1 night).

Percolation is the filtering process of submersion result with the drop way, thus able to gain good extract with the right measurement. The result of percolation put into the evaporation flask 1 L set into the evaporator. Water bath filled water till full with temperature 90°C or based on the boiled solvent level then connected the instrument with electricity current. The solvent will separate with active substance; let it drop until stop to the flask collector approximately 1–2 h. Then, the extracted result put into the glasses bottle. The resulted extract was freeze dried in the biology laboratory. The gained substance in the form of gel 15, 273 g.

### Active compound identification test (coclaurine) from leaves of *C. barbata* extract

Test was done to know the coclaurine compound based on the weight of standard molecule of coclaurine used liquid chromatography-mass spectrometry (LCMS) method. First, the ethanol extract were mixed into tartrat acid and 3% water (1: 1, v / v) to give liquid fraction of acid EtOAc (2,88%) and acid liquid condition. Liquid pH adjusted into nine with saturated water and then extracted with  $\text{CHCl}_3$  to get solutal fraction  $\text{CHCl}_3$  (0,97%). Watery layer extracted furthermore with n-BuOH to get fraction that is easy to soluble n-BuOH (0,62%) through the separation of polarization bioassay.

### Collection compound content of *C. barbata* in the database

Metabolite secondary compound from *C. barbata* loaded from KNApSACk ([http://kanaya.naist.jp/knapsack\\_jsp/top.html](http://kanaya.naist.jp/knapsack_jsp/top.html)) and saved in the format of Molfile. KNApSACk is a database that supplies a secondary metabolite list of plants. There are approximately 50.048 data metabolites which come from 20.741 species of plants which can be useful for metabolomic research [11]. While the protein sample gained from protein data bank (PDB) (<http://www.rcsb.org/pdb/>) in the format of .pdb, PDB is a database for the structure of 3D from various macromolecules biologic such as protein and nucleic acid from various organisms such as human, bacteria, animals, and plants [12].

### Analysis of molecular docking

Active compound structure from *C. barbata* gained from database PubChem (<https://pubchem.ncbi.nlm.nih.gov/search/>) and manual used PubChem Sketcher V2.4 (<https://pubchem.ncbi.nlm.nih.gov/edit3/index.html>). 3D structure protein estrogen receptor gained from PDB database (ID 1A52) and estradiol (EST) as the ligand references. Natural compound which has the most potential and target protein which probably interacted then studied in detail the interaction used program molecular docking PyRx0.8. Docking process used AutoDock Vina and did specifically to the active side of protein target. Molecular docking can predict the affinity of bonding a compound to the particular target protein [13]. Higher affinity potency of a drug will become better. Molecular docking will use to study the mechanism of inhibition/activation a compound through a particular target protein. The complex result of docking analyzed further related to amino acid involved in interaction used the program od LIGPLOT+ and all visualization process used Discovery Studio 2017, thus all displaying data will be more representative.

### Identification of bioactive of *C. barbata* interaction with receptor estrogen $\alpha$

To know the interaction molecular of bioactive compound with protein target, an analysis did by STITCH. Bioactive compound *C. barbata* input to the software. STITCH able to integrate chemical compound and protein based on database paper publication. Some parameter action showed in the program of STITCH such as activation activity, binding, and inhibition and showed with the score. Score approach to 1 showed accuracy interaction in the network [14].

### Prediction of protein targets

The most potential active compounds then analyzed the protein targets used HIT-PICK (<http://mips.helmholtz-muenchen.de/hitpick/>). The approach

used the targeted-focused library, which used to know the protein potency into a target from a new compound. The principle used to get similarity of group function and structure of a new compound with the compound had to find out the protein target [15]. The protein target then analyzed the pathway of the working mechanism. Thus, able to study what kind of biological process that will disturb if the drug bond with the protein target.

### Isolation and follicular culture

The material used was a goat ovary taken from the RPH Sukun unit of Malang, brought to the laboratory by being put into a water bath that was filled with a 0.9% physiological NaCl solution that had been added with 100 IU/mL penicillin antibiotics (Sigma-Aldrich, St. Louis, MO, USA) and 0.1 g/mL streptomycin (Sigma-Aldrich) with temperatures of 35–37°C. Furthermore, the ovaries are cleaned of attached fat tissue [16].

The dosage used in PMSG and hCG for follicular development start from the secondary follicular to pre-ovulation follicles. Maturation medium used tissue culture medium-199, serum supplementation (FBS 10%), paraffin oil added to the dosage group of *C. barbata* extract, and coclaurine dose group. Culture plates have been given PMSG + HCG so that they are conditioned to the actual environment. The maturation of oocytes was

then analyze using an inverted microscope and a stereo microscope with  $\times 200$  magnification [17], [18].

## Results

### The identification and characterization of coclaurine to *C. barbata*

To know the coclaurine compound based on the weight of the standard molecule of coclaurine used LCMS method.

Trial with Frg 286 20 used LCMS method, showed the presence of the target compound of coclaurine with code: 286 inside leaves extract of *C. barbata* (Figure 1); however, there was another compound assumed also has a role in stimulating target.

### The analysis interaction of bioactive compound *C. barbata* with *ESR $\alpha$*

Database showed that there was an active substance in *C. barbata*. To analyze the molecular interaction which occurs in the active compound and target protein, the molecular docking approach was performed.

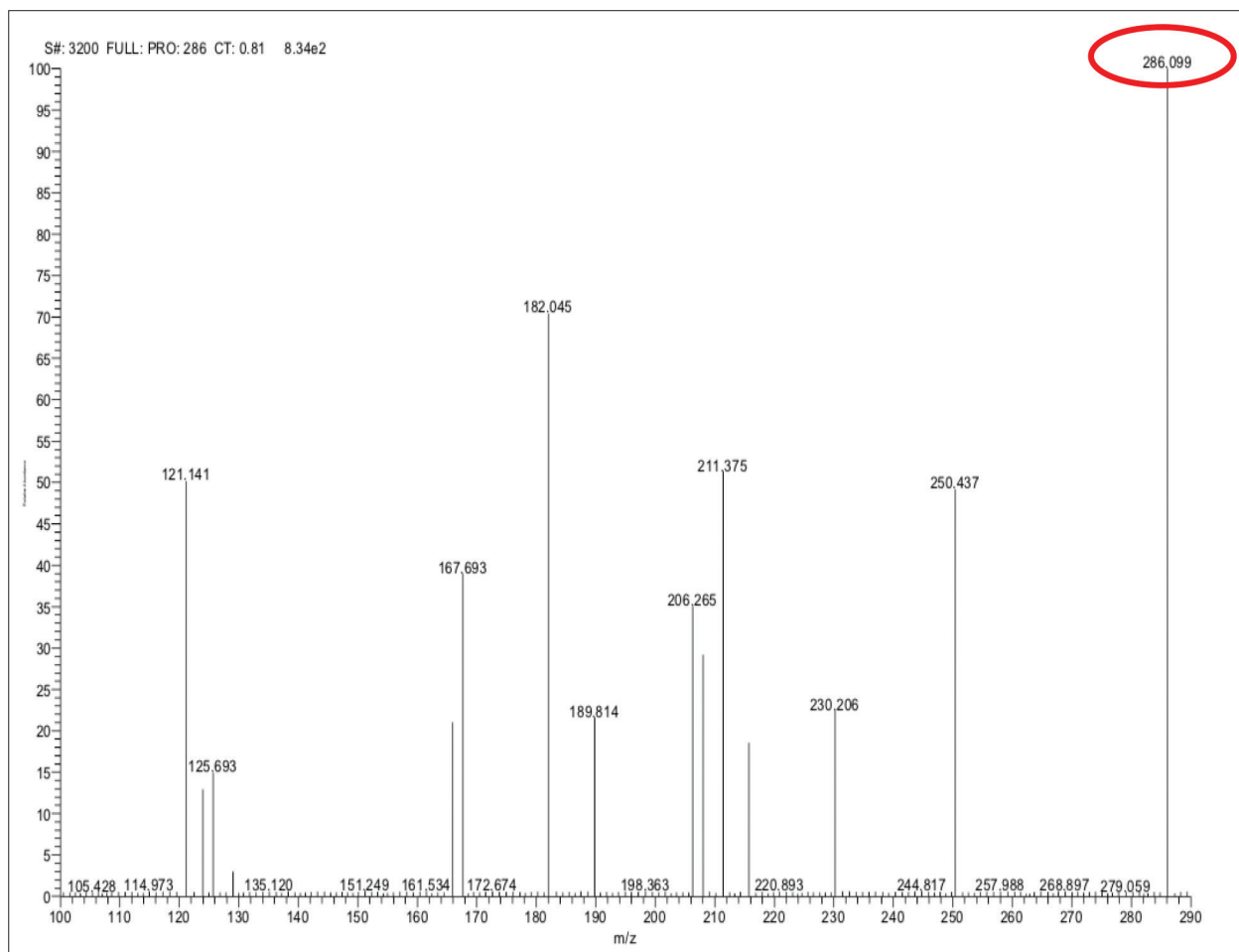


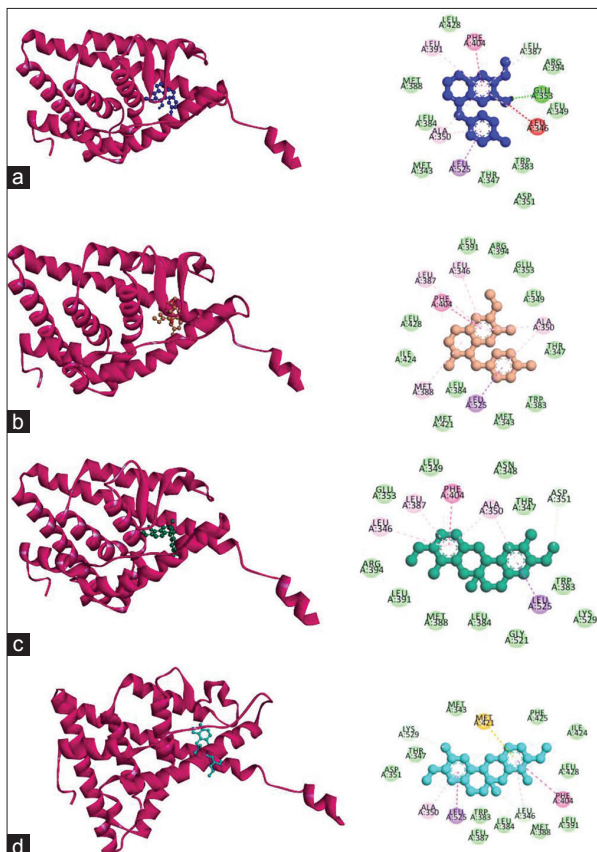
Figure 1: Coclaurine compound detected to data of liquid chromatography–mass spectrometry leaves extraction of *Cyclea barbata* (red circle)

The docking score result shows in Table 1. Predicted there was four main active compounds which has potency to bind with estrogen receptor  $\alpha$ , those were coclaurine; n-methylcoclaurine; cyclanoline; and beta-cyclanoline. The result of docking showed that complex n-methylcoclaurine and coclaurine have the highest binding affinity power, almost similar with the control that was complex estradiol-ESR with score -7.1 and 8.3 kcal/mol continually (Table 1).

**Table 1: The result of docking 18 compounds *Cyclea barbata* with ESR $\alpha$**

Receptor	Ligand	CID	Binding affinity (kcal/mol)
ESR (PDB ID 1A52)	Estradiol (control)		-10.9
	Coclaurine	160487	-8.3
	N-methyl Coclaurine	440584	-7.1
	Cyclanoline	3082134	-3.4
	Beta cyclanoline	5316230	-2.9
	Cycleanorine	3082494	31.3
	Repandine	10031631	32.3
	Thalrugosine	100257	33.1
	Cycleadrine	321937	33.2
	Cycleapeltine	3084718	33.4
	Homoaromaline	9851833	36.1
	Cycleabarbantine	192607	37.6
	Norlimacine	192608	38.8
	Fangchinoline	73481	38.9
	Limacine	100257	40.9
	Curine	253793	50.3
	Isochondodendrine	197726	54.6
	Tetrandine	-	38.6
	Isotetrandine	457825	42

Visualization of complex interaction showed bioactive substance binding to the same site with control estradiol (EST) showed the involved interaction of some same amino acid; those were ARG394, GLU353, LEU387, LEU525, MET388, and PHE404 (Figure 2 and Table 2).



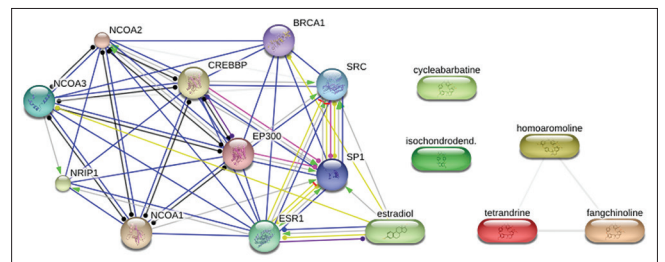
**Figure 2: Visualization complex of interaction estrogen receptor (cartoon, magenta) and compounds (stick and ball): (a) Coclaurine (blue), (b) methyl coclaurine (peach), (c) cyclanoline (tosca), and (d) beta cyclanoline (cyan)**

**Table 2: Four main compounds which have the highest binding affinity and amino acid interaction that involved**

No	Complex	Binding affinity (kcal/mol)	Amino acid interaction
1	ESR – Estradiol (control)	-10.9	ARG394, LEU391, GLU353, LEU387, LEU525, HIS524, ILE424, MET388, LEU48, PHE404
2	ESR – Coclaurine	-8.3	LEU346, LEU391, ARG394, LEU387, GLU353, PHE404, MET388, ALA350, LEU525, TRP383, THR347, LEU349
3	ESR – Methyl coclaurine	-7.1	MET388, LEU384, ARG394, GLU353, LEU387, PHE404, LEU346, LEU525, LEU349, ALA350, TRP383, THR347, MET421
4	ESR – Cyclanoline	-3.4	THR347, ALA350, LEU525, ASP351, TRP383, LEU384, MET388, LEU346, LEU387, GLU353, LEU391, ARG394, PHE404
5	ESR – Beta cyclanoline	-2.9	LEU387, TRP383, LEU428, LEU346, PHE404, PHE425, MET421, ILE424, LEU525, THR347, LYS529, ASP351, ALA350, LEU384

**The interaction network analysis of bioactive with estrogen receptor  $\alpha$**

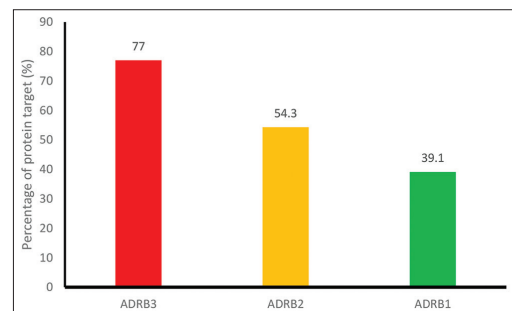
The pathway that happened between estrogen receptor  $\alpha$  and coclaurine compound needs to analyze further, thus find out that cascade signaling occurred. The network result analysis showed that there was no direct interaction between bioactive compound with estrogen receptor  $\alpha$ , signaling by the deformed of connector lines. (Figure 3). These directed to indirect interaction between compounds with the connected protein with the estrogen receptor. There was a probability that coclaurine compound was pass through some proteins before binding with ESR $\alpha$ . Then, it needs to do an analysis of protein target by each compound from the extract.



**Figure 3: Network analysis of STITCH between the compounds of bioactive *Cyclea barbata* with ESR $\alpha$**

**Prediction analysis of networking between protein target and ESR $\alpha$**

To know the correlation between protein target as the result of prediction HITPICK with ESR $\alpha$ , do the analysis of networking protein to protein (Figures 4-7).



**Figure 4: Prediction of main protein target of coclaurine compound**

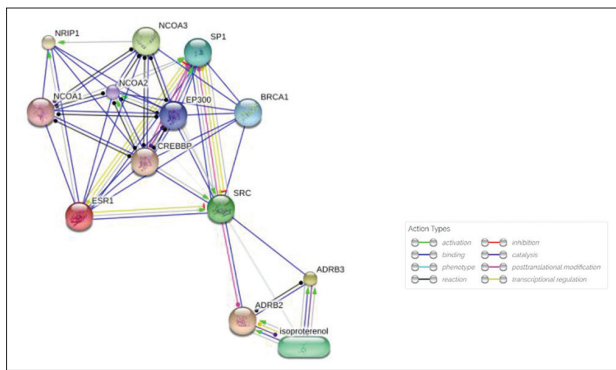


Figure 5: Interaction prediction ESR $\alpha$  to protein

To know the level of maturation to some doses of *C. barbata* extract and coclaurine can be done by observed induce and formation of perfect cumulus expansion and polar bodies in oocytes. Observation was performed using 200 $\times$  inverted microscope and stereo microscope. The results are as follow:

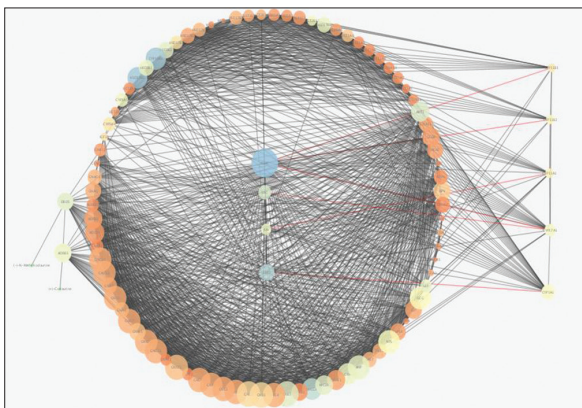


Figure 6: The pathway involved in biosynthesis of the steroid

Based on Figure 8a explained that the sum of maturation to dosage 100 has a higher maturation level that was 3 (75%), while to dosage 25 and 50 only gained score 1 (25%).

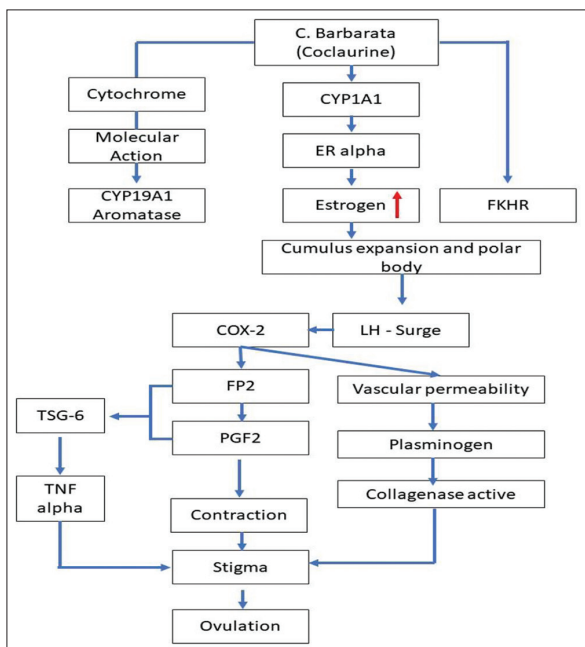


Figure 7: The summary of pathway passed through by coclaurine at the time of interaction with estrogen receptor  $\alpha$ .

Based on Figure 8 explained that the sum of maturation to dosage 100 and dosage 200 had the same score was 2 (50%), while to dosage 50 only gained score 1 (25%).

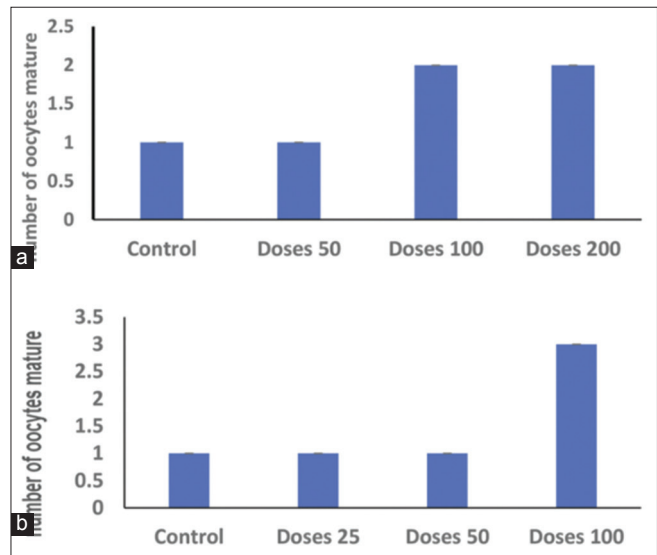


Figure 8: a) The description of oocyte maturation which has given *Cyclea barbata* ethanol extracts dosage; b) The description of oocyte maturation which has given coclaurine dosage

## Discussion

This study finding that N-methyl coclaurine and coclaurine have binding potential to ESR $\alpha$  which is estradiol receptor. Estrogen has an important function in regulating the delivery process in various aspects [19].

Estrogen is not only work alone but also cooperate with some hormones which have role in the delivery process that is progesterone. Biosynthesis progesterone and mechanism transfer of placenta able to induce the maturity of fetal hypothalamic-pituitary-adrenocortical axis [20]. Although in the end of the development process directed to the final gestation, so few information known on the role of placenta trophoblastic estrogen during the early gestation process. The newest review explained that estrogen has main role to induce the development of placental blood vessel and fetal ovarian folliculogenesis during gestation.

Indirect connection formed between coclaurine with ESR $\alpha$  directed to the hypothesis that coclaurine passes some cascade signaling before influence the activity of ESR $\alpha$ . To know the main target of protein from coclaurine did prediction analysis used HITPICK. The result showed that there were two main targets such as protein ADRB3 and ADRB2, with each accuracy were 77% and 54.3%. Both of the proteins targets have score tanimoto coefficient (Tc) above threshold 0.5 (Figure 4). Those showed that coclaurine compound has structure similarly with a chemical structure that has a publication and has a similar protein target.

ADRB2 or adrenergic receptor beta-2 is membrane cell that binding epinephrine, hormone, and neurotransmitter, and signaling through the stimulation adenylate cyclase. To women reproduction cycles, luteinizing hormone receptor stimulates adenylate cyclase in producing sterol through cAMP then the sterol will change into hormone estrogen through molecular action metabolism steroid by cytochrome aromatase (Cyp19A1) [21], [22], while ADRB3 or adrenergic receptor beta-3 activated ESR $\alpha$  thus caused the increase of estrogen hormones through metabolism steroid by Cyp1A1. As we know that the group of cytochrome is able to stimulate steroid metabolism through the activation of G protein in the cell surface [4], [23]. The increase of estrogen will cause the occurring of LH surge that becomes the early step of ovulation/the maturation of egg sell to women. If at that time, there is an insemination; thus, there will be a gestation or pregnancy [4], [23].

The correlation between protein targets as the result of prediction HITPICK with ESR $\alpha$ , do an analysis of networking protein-protein. The result showed that ADRB2 and ADRB3 have binding firstly with SRC, showed that the blue line formed (Figure 5). The action then affected to the ESRalpha expression as blue and yellow lines formed. The involvement of SRC considers important and priority, showed by the result of Cytoscape (Figure 5). SRC knows that it has a role in regulating the biology activity such as gene transcription, immune response, cell adhesion, cell cycle progression, apoptosis, migration, and transformation to some signaling pathway [21], [22].

The importance of protein SRC in the pathway does an analysis use Cytoscape (Figure 6). Based on the result, assumed there is a potential path of coclaurine with ERS $\alpha$ , SRC is one of the main paths that occurred in the interaction of ESR $\alpha$ . Cytochrome (CYP) which is playing role in the biosynthesis steroid has many possible pathways, including SRC pathway (Cyp17A1), POMC pathway (CYP11B1, 11B2, 17A1), JUN pathway (CYP11A1), and ESRalpha pathway (CYP1A1). CYP19A1 has a great role; however, *in silico* has not found the pathway especially.

The summary pathway that occurred in the menstruation cycles involved the giving of coclaurine compound as the induction of estrogen hormone. Coclaurine gives stimulation of estrogen hormones induction through some proteins those are SRC, ADRB2, and ADRB3 which involved CYP1A1 and CYP19A1, and activate estrone and estradiol to help to increase the estrogen (Figure 7). Estrogen hormones are responsible to stimulate ovulation in each menstruation cycle. Estrogen is very influence for the occurring of ovulation through granulose cell, enzyme aromatase, and theca cells. The increase of estrogen in the follicle influenced by the synthesis from FKHR and also the presence of role CYP19A1 aromatase which changes steroid in the case of testosterone and androstenedione into estrogen [22].

The process of steroidogenesis which occurs in theca cells started from cholesterol take to the outer membrane of mitochondria and moved to the inner part of mitochondria membrane by protein steroidogenic acute regulatory (StAR). Then, the cholesterol is converted to pregnenolone by enzyme cytochrome side-chain cleavage (P450scc). Pregnenolone diffuses out of the mitochondria and into the reticulum of endoplasm and is converted into progesterone by 3 $\beta$ -hydroxysteroid and becomes testosterone by 17 $\beta$ -hydroxysteroid 2. Besides that, pregnenolone is converted to become DHEA and then converted into androstenediol by 17 $\beta$ -hydroxysteroid 1. Testosterone and androstenediol called also androgen which becomes the basic material of estrogen [21], [22].

The deficiency activity of gene expression of CYP19A1 aromatase causes a decrease of estrogen. At the time of dominant follicle/tersier follicle has reached maturity, estrogen secretion resulted enough to create "positive feedback" that caused by the secretion of hormone concentration of LH in high doses from the pituitary gland (LH surge) [4]. LH surge is the positive feed result from the increase of estrogen in intracellular which impacts to ovulation. The increase of ovulation is influenced by the presence of LH surge. LH surge occurred caused by the biosynthesis process which weakens the follicle wall that ended by the production of oocyte from the follicle [4], [5].

LH induced COX-2, which function to make expansion of cumulus and ovulation. COX-2 activation by LH is necessary for expansion and ovulation of the cumulus. COX-2 is essential for prostaglandin synthesis, that is, PGE2 which binds the EP2 receptor. LH COX-2 induction can be direct or indirect through activation of the oocyte-derived factor, growth differentiation factor (GDF)-9. Mice null fail to ovulate and show defective cumulus cell expansion for COX-2 and EP2, a mechanism that involves the synthesis and cross-linking of main components of the matrix. Which are hyaluronic acid (HA) and protein binding HA, inter-alpha-inhibitor (IalphaI), and gene suppressor of tumors (TSG)-6. IalphaI is supplied by serum, and reaches follicles when the basement membrane is removed in reaction to LH. TSG-6 is induced by LH in the cumulus cell of follicle ovulation; induction of TSG-6 depends on induction of COX-2 and expression receptor EP2. TSG-6 will induce the occurring of apoptosis which impacts to the death of a cell in the follicle area and supporting stigma to break and releasing oocyte [24].

*C. barbata* ethanol extract and coclaurine have proved able to give maturation effect of oocyte to secondary follicle. If compared with control, the increase of maturation gained from giving *C. barbata* extracts with dosage 100 and coclaurine with dosage 100 and 200. From this testing proved that a higher dosage of *C. barbata* ethanol extract and coclaurine is given thus it will be higher the effect of maturation.

## Conclusion

*C. barbata* ethanol extract and Coclaurine have proven to give maturation effect of oocyte to secondary follicle. Higher doses of *C. Barbata* ethanol extract and Coclaurine positively effect the follicle maturation.

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