Purification and characterization of water soluble natural compounds from the marine algae Padina that interact with the membrane progesterone receptor (mPR)

Thesis

Purification and characterization of water soluble natural compounds from the marine algae *Padina* that interact with the membrane progesterone receptor (mPR)

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CONTENTS

LIST OF ABBREVIATION

AgNPs	: Silver nanoparticles
17α, 20β-DHP	: 17α, 20β-dihydroxy-4-pregnen-3-one
DES	: Diethylstilbestrol
EtOH	: Ethanol
EDTA	: Ethylenediaminetetraacetic acid
cAMP	: Cyclic adenosine monophosphate
DNA	: Deoxyribonucleic acid
DDW	: Double distilled water
GPCR	: G protein-coupled receptor
GVBD	: Germinal vesicle breakdown
LH	: Luteinizing hormone
GTH	: Gonadotrophic hormone
MAPK	: Mitogen-activated protein kinase
MIH	: Maturation inducing hormones
MIS	: Maturation-inducing steroids
MPF	: Maturation promoting factor
mRNA	: Messenger RNA
FSH	: Folic stimulating hormone
g	: Gram
HPLC	: High performance liquid chromatography
mPRα	: Membrane progesterone receptor alpha
mPRβ	: Membrane progesterone receptor beta
mPRγ	: Membrane progesterone receptor gamma
mPRδ	: Membrane progesterone receptor delta
mPRϵ	: Membrane progesterone receptor epsilon
mM	: Millimolar
μL	: Microliter
nPR	: Nuclear progesterone receptor
nm	: Nano meter
PR	: Progesterone receptor
PGRMC1	: Progesterone receptor membrane component 1
PGRMC2	: Progesterone receptor membrane component 2
PKA	:Protein kinase A
PKC	: Protein kinase C
CaMKIV	: Calcium/Calmodulin-dependent protein kinase
PI3K	: Phosphatidylinositol-3 kinase
PCR	: Polymerase chain reaction
PBS	: Phosphate buffered saline
PtNPs	: Platinum nanoparticles
Rpm	: Revolution per minutes
Spp.	: Species
^{O} C	: Degree conscious

EXECUTIVE SUMMARY

Marine algal flora significantly contribute in the productivity, flow of energy in the ecosystem, remediation of contaminants and nutrient cycling between terrestrial and aquatic ecosystems. Moreover, marine algae also release some chemical, which has some hormonal like activity. As we know that progestins are the synthetic forms of the hormone progesterone, which occurs naturally in the body and acts a very significant role in controlling reproduction. The physiological effects of progesterone are mediated through modulating the expression of genes linked with nucleus progesterone receptors (nPRs), but the discovery of membrane progestin receptors (mPRs) brought new insights into progesterone action. Scientists are now focusing their efforts on identifying the new mPR ligand. Previously, our laboratory group conducted one of the research projects on detecting the membrane progestin receptor (mPR) interacting compound of coral sea water, for which they collected samples from Mauritius. Other mPR ligands, such as DES, DHP, and Org OD 02-0, were discovered in early research after the discovery of membrane progestin receptors (mPRs) as a nongenomic signaling pathway.

Current study attempted to cultivate the marine algae known as "*Padina*" in *in-vitro* condition to collect the natural active compound. The samples were collected from the at marine field of Shimoda Marine Research Center, University of Tsukuba or Mochimune marine filed of Faculty of Agriculture Regional Field Science Education and Research Center, Shizuoka University. All the samples were transported into aquarium in the day of sampling. Algae were planted in gravel stone in 120 cm long aquariums and cultivated for several months in culture room of Mochimune marine filed, Shizuoka University. Filtering systems and LED lights were set on the aquariums. Firstly, the species of the *Padina* was identified by DNA sequencing. The Molecular analyses using mitochondrial *cox3* genes as molecular markers confirmed the species as *Padina arborescens*. The secreted compounds were accumulated by filtration system and the compounds were collected from the filter by using the absolute ethanol. Then the samples were concentrated in the ODS column by peristaltic pump. High performance liquid chromatography (HPLC) was implemented to separate the compound from column. The samples were fractionated with four different colors. Several steps of HPLC analysis were accomplished to obtain the final refined samples. The binding affinity of the samples were significantly potent towards the mPRα, which was governed by the steroid binding assay with the crushed cells membrane of transfected cell comprising the mPRα gene. Finally, fraction with mPRα-interacting activity was purified by TSKgel Phenyl-5PW RP Glass with two peaks. The existence of specific chemical compound in the samples were unveiled by ESI mass spectrometry. According to the major signal, the molecular mass was recorded for peak 1

sample as 554 Dalton while the compound of peak 2 showed the molecular mass 554 and 437 Dalton. The fluorogenic attributes of the purified compounds were also determined through spectrometric analysis, the absorbance and fluorescent scanning pattern at maximum excitation indicated that the peak 1 was excited at 418nm and emitted at 668nm while the peak 2 was excited at 412nm and emitted at 672nm. Meanwhile, the purified chemical exhibited the antagonistic attributes with 17α, 20β-dihydroxy-4-pregnen-3-one (17,20β-DHP) in order to induce the zebrafish oocyte maturation and ovulation *in-vivo*. The purified compounds were unable to induce the fish oocyte maturation and ovulation. On account of this, we attempted to evaluate their antagonistic activity on inducing activity by natural hormone, 17,20β-DHP. In case of *in vivo* assay, oocyte maturation and ovulation was inhibited significantly by both compound though the oocyte maturation was not prevented completely by the compound from peak 2. Meanwhile, the oocyte maturation was also inhibited by *in vitro* assay for both compounds.

Based on the current findings, it can be concluded that two secreted compounds from *Padina* were purified and characterized in this study. Purified compounds interact with the membrane progesterone receptor, or mPR. *In vitro* and *in vivo* assay revealed that the compounds inhibit fish oocyte maturation and ovulation outstandingly. Our purified compounds were highly fluorogenic. However, determination of the chemical structure of the compound which has not yet successful would be our future prospect as new pharmaceutical candidate as well as to make the compound commercially available.

General Introduction

Marine algal flora significantly contributes in the productivity, flow of energy in the ecosystem, remediation of contaminants and nutrient cycling between terrestrial and aquatic ecosystems. Micro and macroalgae are sensitive to environmental factors and indicate the ecological status and potential of marine ecosystems. Moreover, marine algae also release some chemical, which has some hormonal like activity especially steroidal which has been considered as their pharmaceutical and medical importance. As we know that progestins are a class of steroid hormones that act as key-regulating factors controlling reproduction. The physiological properties of such progesterone are facilitated by regulating the expression of genes those are associated with nuclear progesterone receptors (nPRs). After discovering the membrane progestin receptors (mPRs), scientists have found novel perceptions regarding the progesterone activity.

Previously, one of our research group became successful to perceive the presence of a novel steroid membrane receptor (mPR) interacting natural hormone active substance first time from Mauritius coral reef seawater (Tokumoto et al 2017). To enhance the reproductive biology and hormone research marine ecology would be the best sector to discover the new mPR ligand as the natural steroid compound.

Marine algae *Padina* as the organisms

Macroalgae are widely recognized as an important source of food in many Asian countries. In today's world, macroalgae are gaining popularity as a valuable food source (Mac Artain *et al*. 2007). So far, approximately 221 macroalgal species have been reported for commercial use around the world (Zemke-White and Ohno 1999). Algae is widely used for human food, fodder, fertilizer, drugs, paper production, and a variety of other industries, the majority of which are devoted to the extraction of phycocolloids and fine biochemicals. A few *Padina* species have traditionally been used as a food source in many coastal areas around the world. *Padina* has also been reported to be used as a salt substitute in the treatment of high blood pressure patients, as well as in the treatment of other diseases such as goiter and scrofula (Novaczek and Athy 2001, Anggadiredja 1992).

Systematic Position of *Padina* Division: Phaeophyta
Class: Phaeophyceae
Order: Dictyotales

Family: Dictyotaceae Genus: *Padina*

Structure and Description of *Padina*

Padina thalli (fronds) are flabellate, with a thin deposit of lime giving them a brown or whitish appearance. Thalli are frequently conspicuously zonate, with compacted rhizoidal holdfasts connecting them; the stipe is frequently invested with rhizoids. Blades are two to several celled thick, with zones denoted by concentric rows of hairs, which are sometimes divided into narrow spatulate segments. Side to which blades are inrolled is called the upper surface of the blades (Fig. 1). *Padina* has a diplo-haplontic and isomorphic life cycle. Gametophytes can be dioecious or monoecious, with reproductive cells such as sporangia and gametangia scattered on blades or in sori between thalli hair bands. (Geraldino *et al.* 2005).

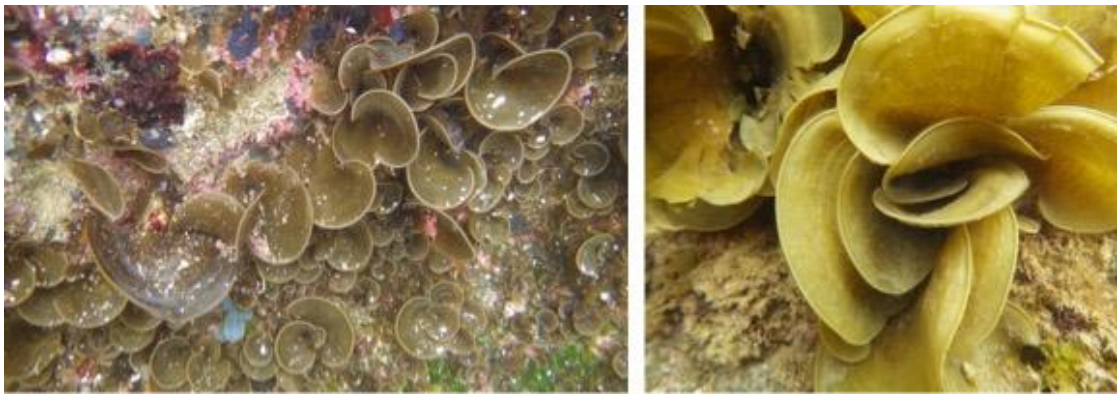


Figure 1. Photograph from the natural habitat of *Padina* spp.

Ecology and Distribution

Padina species are widely distributed throughout the tropics and are very easy to identify in the field. Infrageneric segregation, on the other hand, necessitates microscopic morphological confirmation. *Padina* is found in a wide variety of habitats, from the intertidal zone to the subtidal zone. *Padina* sp. is abundant in clear waters to a depth of 15-20 m. They grow attached to solid substrates or as epiphytes on large seaweeds such as Sargassum spp. They are more plentiful during the months of full sunlight. *Padina is widely distributed throughout the tropics and is easily* identified due to its structure's resemblance to a peacock tail (Subramanian *et al.* 2015).

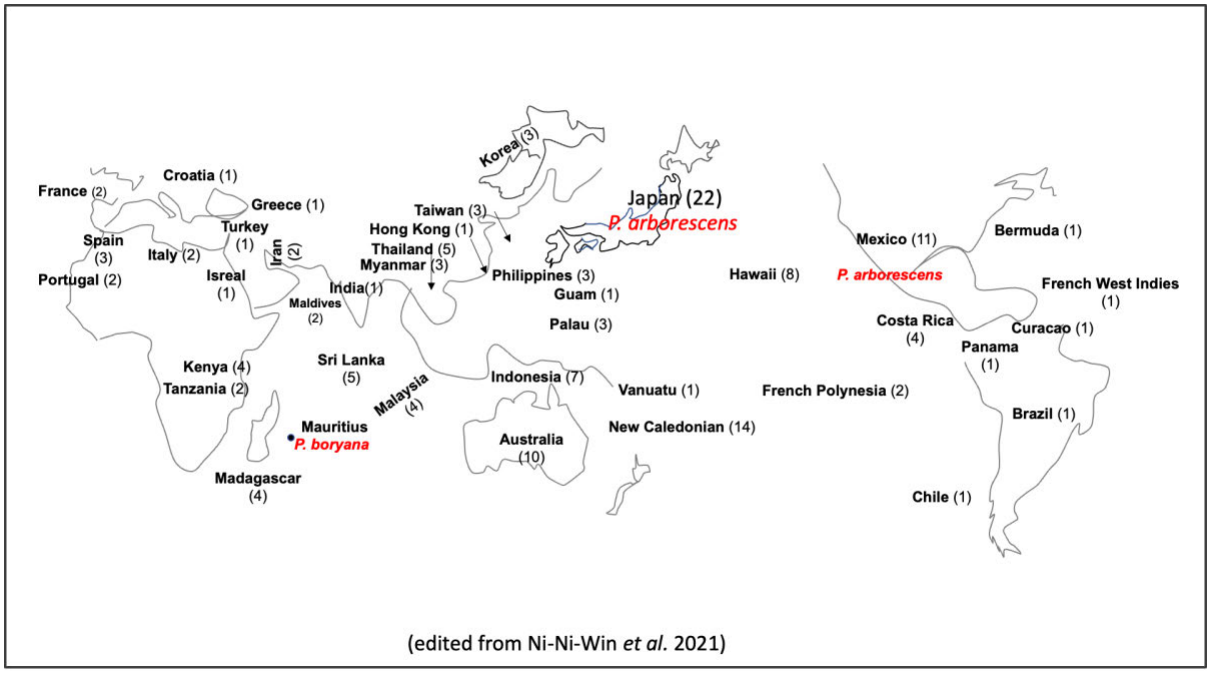


Figure 2. Global species diversity and occurrence of *Padina*. This map indicating the different *Padina* species are now available in worldwide. In Japan, 22 species of *Padina* has already identified.

Padina is found in South America and Southeast Asia, ranging from the tropics to cool temperate waters. So far, 43 *Padina* species have been identified. (Fakhry 2013). Relatively high species richness occurs along Pacific coasts (Japan, 22 species; New Caledonia, 14 species; Mexico, 11 species; Australia, 10 species; Hawaii, 8 species; Indonesia, 7 species). Other regions, such as the Mediterranean Sea, Southeast Asia, the northern Indian Ocean, and the Caribbean Sea, have lower species richness, with 1 to 5 species (Fig. 2). *Padina* species that have recently been discovered are mostly found in Japan, particularly in the southern regions. The distributions of species along the Pacific coasts overlap somewhat, whereas those from the Mediterranean Sea, Indian, and Atlantic Oceans tend to be confined to their respective regions. (Win *et al.* 2021).

Importance of *Padina* in different sectors:

Nanoparticles such as AgNPs and PtNPs are gaining popularity due to their appealing physicochemical properties for a variety of applications. The synthesis of nanomaterials from seaweeds and their various applications is a current area of research interest. Among the noble metals, nanoparticles are highly valued for their catalytic, antimicrobial, hemolytic, cytotoxic,

and antioxidant properties, as well as their low toxicity. AgNPs can be made by reducing aqueous silver nitrate ($AgNO_3$) solutions with powder and solvent extracts of *Padina*. Similarly, PtNPs are synthesized from *Padina* aqueous extract. (Ansari *et al.* 2019). The presence of 19 terpenes and 5 sterols in brown algae *Padina* was discovered through qualitative and quantitative estimation of terpenes and sterols. *Padina* had a high content of ashes (30-48%), carbohydrates (25-39%), and total dietary fiber (27-39%) on a dry basis, 5-7% protein, and 1.6-1.8 percent lipids. Fatty acids with 14–22 carbon atoms have also been discovered in various *Padina* species. Seaweeds also contain polysaccharides, minerals, and vitamins. Seaweeds are used in cosmetics to protect against radiation and in pharmaceuticals as antioxidants, antibiotics, anti-inflammatory, hypoallergenic, antibacterial, and anti-diabetic agents (Ansari *et al.* 2019). *Padina* macroalgae produce antibacterial compounds that are important in inhibiting the growth of various pathogens in humans and other animals. Padina has a high potential for producing novel antibacterial compounds, either alone or in collaboration with antagonistic bacteria (Ansari *et al.* 2019).

Seaweeds are well known for their medicinal value, as reported by numerous researchers from around the world. They are a potential source of biologically active metabolites that are not found in other organisms and are used in the pharmaceutical and pharmaceutical industries. These antioxidant compounds are produced in their body tissues as a reaction to the harsh environmental conditions in which they live. *Padina* extracts, which are hepatoprotective, hypolipidemic, antioxidant, and anti-inflammatory, can be used to treat hepatotoxicity (Ansari *et al.* 2019).

Padina, a brown seaweed, can also be used as an organic fertilizer. *Padina* aqueous extract is high in macronutrients such as nitrogen (N), phosphorus (P), and potassium (K), all of which are necessary for plant growth and development. When used as a bio stimulant, a lower concentration of *Padina* extracts affects germination rate, cell division, and protein pattern. Because seaweeds can undergo biological magnification, they can be used as a bioorganic eco-friendly fertilizer to stimulate the growth of a variety of economically important crops rather than synthetic chemical fertilizers that are harmful to the environment. The use of modern agriculture in conjunction with traditional farming practices is the long-term solution. Several researchers have proposed that brown seaweed liquid fertilizer (BSLF) can be used at low concentrations to improve seed germination and biochemical constituent accumulation in various crop plants (Ansari *et al.* 2019).

Algae are crucial in environmental monitoring and management. In all aquatic ecosystems, they are the primary producers. They provide the energy required for aquatic ecosystems to function. They react quickly to even minor changes in their surroundings. *Padina* has been reported to have a high adsorption capacity to various pollutants on its fresh and dried biomass. They are good indicators for the dynamics of various factors in the aquatic environment, such as light, temperature, nutrients, and a variety of other contaminants. With increasing Cd concentrations, *Padina* (Phaeophyta) showed a decrease in specific growth rate and Chlorophyll content, indicating Cd pollution. *Padina* and other macroalgae are ecologically significant because they can be used as biological indicators for environmental contamination and in the remediation of coastal marine ecosystems. (Ansari *et al.* 2019).

Progesterone and its impact

Progesterone is an endogenous steroid hormone produced primarily by the adrenal cortex and the gonads, which include the ovaries and testes. During the first ten weeks of pregnancy, the ovarian corpus luteum secretes progesterone, followed by the placenta later in the pregnancy. Progesterone generation from the corpus luteum to the placenta usually occurs after week ten. (Kumar *et al.* 2012). Progesterone is a cholesterol derivative with numerous functions in the human body, particularly in the reproductive system (Goletiani *et al.* 2007). Progesterone's primary action is thought to be mediated by its interaction with the intracellular nuclear progesterone receptor (nPR). However, there has recently been evidence to support a role for plasma membrane progesterone receptors (mPR). Different receptors, such as nuclear progesterone receptors (nPRs) and membrane progestin receptors, regulate the expression of various genes associated with progesterons (mPRs). Indeed, the mPRs are 7-transmembrane proteins that contain the PAQR family's seventh gene, including mPR (PAQR7), which is one of the protein subtypes implicated in physiological functions in reproductive tissues (Thomas and Pang 2013).

Membrane progesterone receptors (mPRs)

mPRs are a class of cell surface receptors and membrane steroid receptors that bind the endogenous progestogen and neurosteroid progesterone, as well as the neurosteroid allopregnanolone, to the progestin and adipoQ receptor (PAQR) family (Thomas and pang 2012, Petersen *et al.* 2013) Unlike the progesterone receptor (PR), which is a nuclear receptor whose effects are mediated by genomic mechanisms, mPRs are cell surface receptors that rapidly alter cell signaling through modulation of intracellular signaling cascades (Thomas and

pang 2012). mPRs play important roles in the male and female reproductive tracts, the liver, neuroendocrine tissues, and the immune system, as well as in breast and ovarian cancer. The mPRs appear to be involved in progesterone and allopregnanolone's neuroprotective and antigonadotropic effects

(Thomas and pang 2012, Petersen *et al.* 2013). The progesterone active metabolite 5-dihydroprogesterone, which is also a progestogen, and allopregnanolone, which are positive allosteric modulators of the GABAA receptor, have been shown to rapidly influence sexual receptivity and behavior in mice, actions that are GABAA receptor-dependent (Pfaus 1999, Frye *et al.* 1998) .

Steroid Receptor Crosstalk With Cellular Signaling Pathways

The mPRs are plasma membrane GPCRs that bind progesterone with high affinity. mPRα, mPRβ, and mPRγ are directly coupled to G proteins, where they activate pertussis-sensitive inhibitory proteins (Gi/o) to inhibit adenylyl cyclase activity, cAMP activation, and downstream signaling pathways. In contrast, mPRδ and mPRε activate a stimulatory G protein (G_s) to upregulate adenylyl cyclase activity. The mPR-initiated Gi protein-mediated inhibitory signaling pathway has been shown to reduce steroid receptor coactivator 2 (SRC-2) levels and classical progesterone receptor transactivation, demonstrating membrane-classical PR cross-talk. PGRMC1 and PGMRC2 are found in the preoptic and hypothalamic nuclei, which are important reproductive regions. According to recent research, PGMRC1 acts as an adapter protein for mPRα. Estrogen has been shown to have rapid effects in neural circuits involved in homeostatic functions, reproduction, and energy balance. Estrogen signaling is thought to occur through direct activation of G protein-linked cytosolic signaling kinase pathways (PKA, PKC, CaMKIV, PI3K), resulting in the phosphorylation and activation of numerous proteins, rapid enzyme induction, and neuronal function modulation. Significant evidence suggests that ERα and ERβ are expressed on the membrane surface, share epitope homology with classical estrogen receptors, are localized to caveolae lipid rafts in signaling complexes, and facilitate rapid signaling. Depending on the cellular context, the complex's signaling proteins include G proteins, growth factor receptors, non-growth factor tyrosine kinases, glutamate receptors, linker proteins, and orphan GPCR. Furthermore, GPCR30 (also known as GPER) and Gq-membrane estrogen receptors mediate rapid signaling by modulating Ca^{+2} and K^{+} currents in neurons, respectively. Androgens' fast-acting functions, like estrogen and progesterone, modulate changes in G protein signaling. These rapid androgen actions also

interact with classical androgen receptors via a scaffolding protein, a modulator of nongenomic steroid receptor actions (MNAR). Mineralocorticoids, vitamin D, and thyroid hormones all have fast acting effects that are mediated by membrane-initiated kinase signaling cascades, just like other members of the steroid receptor superfamily (Nancy *et al.* 2017).

Oocyte Maturation

The mammalian ovary is a highly dynamic organ that undergoes numerous structural and functional changes as it performs its two major functions of producing female gametes and synthesizing sex steroids. During fetal life, germ cells (oocytes) form in the human ovary and are enclosed within somatic cells (granulosa cells) to form primordial follicles. The primordial follicles are made up of an oocyte that has been arrested in prophase I of meiosis at the diplotene (dictyate) stage, surrounded by a few flattened somatic cells (granulosa cells). For many years, it was assumed that oocytes can only be formed for a limited time and that the adult ovary has no capacity for germ cell renewal, and that primordial follicles represent a pool of oocytes that must last the woman throughout her reproductive lifespan (Fig. 3). The treatment of ovarian insufficiency and failure, including infertility caused by aging or damage, is largely based on the belief that the entire germ cell (oocyte) pool is present at birth and that ovaries lose their ability to renew oocytes (oogenesis) under physiological and perturbed conditions. For nearly a century, scientists have debated whether the adult mammalian ovary has the ability to undergo postnatal germ cell renewal. Since the 1950s, scientists have believed that the population of primordial follicles is fixed before or around the time of birth, depending on the species. Despite exceptions, the presence of a continuous germline lineage has been identified in the ovaries of prosimian primates for many years. Until recently, the existence of similar cells in adult human ovaries had not been actively studied (Telfer and Anderson 2019)

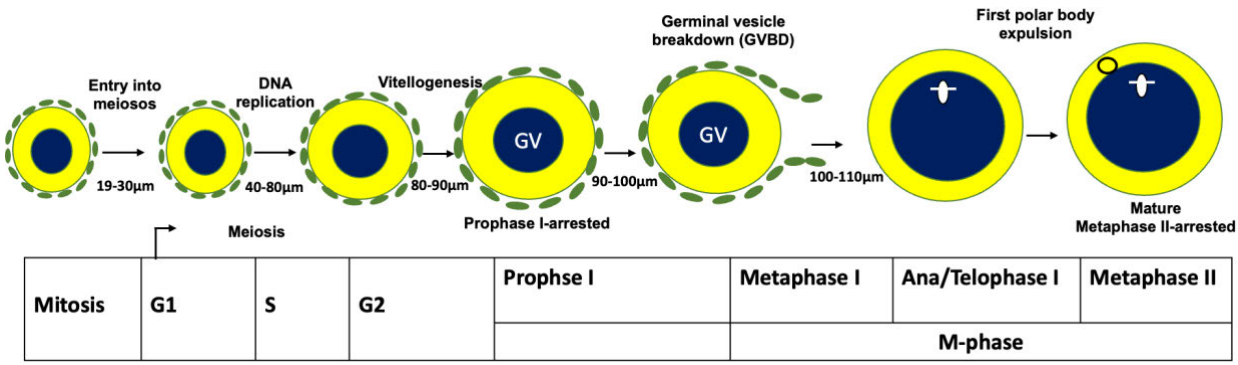


Figure 3: Process of early oocytes development to oocytes maturation. The appropriate development of the oocyte is required for normal female fertility. This development reaches a pinnacle immediately before ovulation when the oocyte matures. Oocyte maturation refers to a release of meiotic arrest that allows oocytes to advance from prophase I to metaphase II of meiosis

Mechanisms of Oocyte maturation

Fishes have had the most extensive research into the endocrine regulation of oocyte maturation. Using well-characterized in vitro and in vivo systems, researchers discovered that oocyte maturation in fish is regulated by three mediators: gonadotropin (GTH; luteinizing hormone, LH), maturation-inducing hormone (MIH), and maturation-promoting factor (MPF) (MPF). Gonadotropin (luteinizing hormone, LH) released from the pituitary stimulates the production of maturation-inducing hormone (MIH) (17α,20β-dihydroxy-4-pregnen-3-one, 17,20β-DHP in most fishes) by the ovarian follicle layers, thecal and granulosa cell layers (Fig. 4).

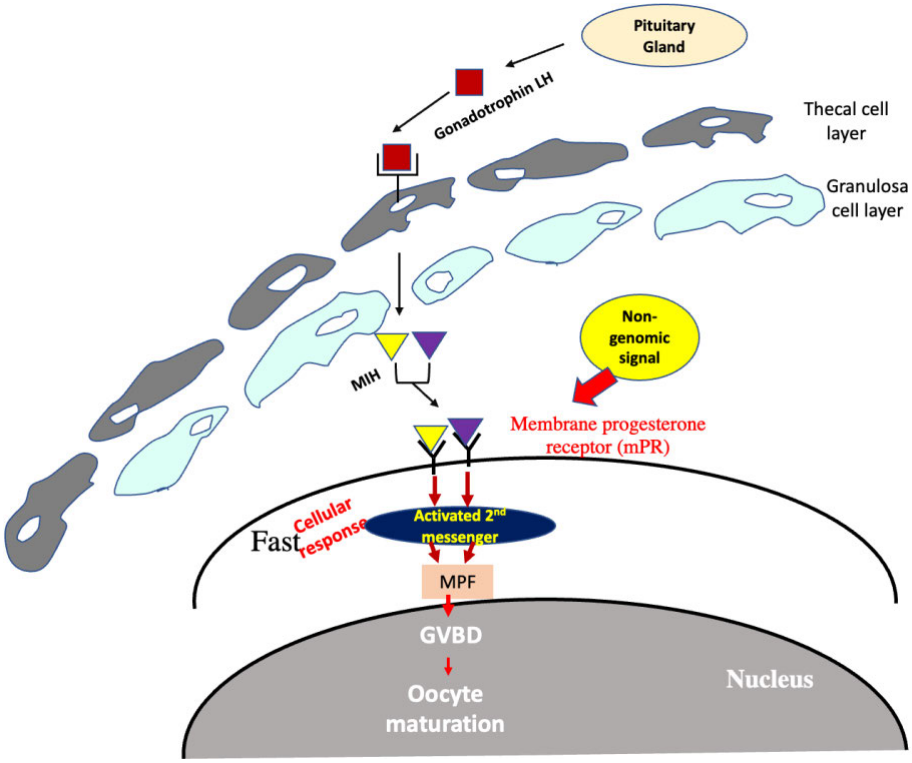


Figure 4: Summary of mechanism of oocyte maturation. The hormonal regulation of oocyte maturation in fish is depicted in this diagram. The pituitary hormone gonadotropin (luteinizing hormone, LH) stimulates the ovarian follicle layers, thecal, and granulosa cell layers to produce maturation-inducing hormone (MIH). MIH's oocyte maturation activity is mediated by a

complicated interplay between oocyte MIH receptors and signal transduction pathways via inhibitory G-proteins.

Meanwhile, MIH action on oocyte maturation is mediated by a complex interaction of oocyte MIH receptors and signal transduction pathways via inhibitory G-proteins. Through a phosphorylated complex of cyclin B and cdc2, the MIH signal induces the formation of maturation-promoting factor (MPF), which then induces germinal vesicle breakdown and oocyte maturation (Nagahama and Yamashita 2008)

Ovulation

The release of eggs from the ovaries is referred to as ovulation. This occurs in women when the ovarian follicles rupture and release secondary oocyte ovarian cells. Ovulation is the timely release of a mature, developmentally competent oocyte from the ovary into the oviduct, where fertilization takes place. Importantly, ovulation is intimately related to oocyte maturation, demonstrating the interdependence of these two parallel processes, both of which are required for female fertility. The ovulatory process is initiated by pituitary gonadotropins and is mediated by intrafollicular paracrine factors from the theca, mural, and cumulus granulosa cells, as well as the oocyte itself. During oocyte maturation, the microvillar connections between follicular cells and the oocyte are expulsed from the follicles via the opening site, known as follicular rapture, and the fertilization membrane is produced (Fig. 6)

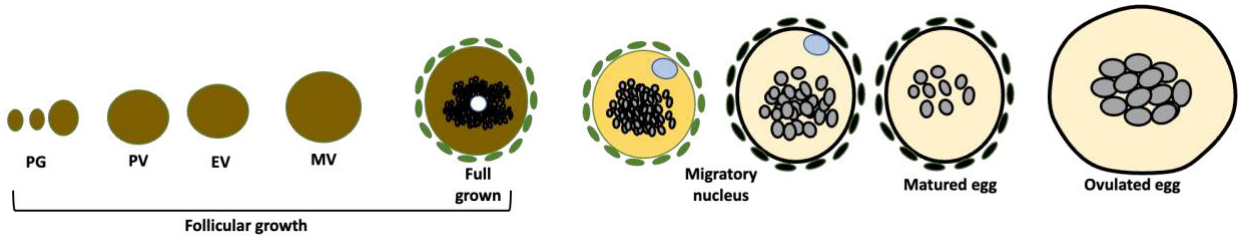


Figure 5: Whole process from oocytes maturation to ovulation. This image is showing the different changes in oocyte during the follicular growth. From the primary growth to ovulation. PG indicating primary growth, PV indicating previtellogenic, Ev indicating early vitellogenic stage.

Oocytes maturation and ovulation through genomic and non-genomic signaling pathway

Several endocrine disrupting chemicals have already been established those may act as a ligand of mPR (non-genomic pathway) and nPR (genomic pathway). The most common and

frequently used chemical such as 17α, 20β-DHP and diethylstilbestrol (DES) initiates oocyte maturation. To induce oocyte maturation, 17α, 20β-DHP bind to the membrane progastrin receptor (mPR) and transduce the signal into the cytoplasm, where the second messenger is activated very quickly and the maturation inducing hormone is activated. The germinal vesicle breakdown eventually occurs, and the oocytes mature. The 17α, 20β-DHP can also bind to the nuclear progastrin receptor (nPR). The information is passed through mRNA in this pathway, and ovulation occurs as a result of the gene being expressed. DES, on the other hand, is a non-steroid estrogen that can only stimulate oocyte maturation via the mPR (Tokumoto *et al.* 2004). Among the two pathway, nPR oriented signal (genomic pathway) can pass very slowly than the mPR (non-genomic pathway) oriented signal (Fig. 6).

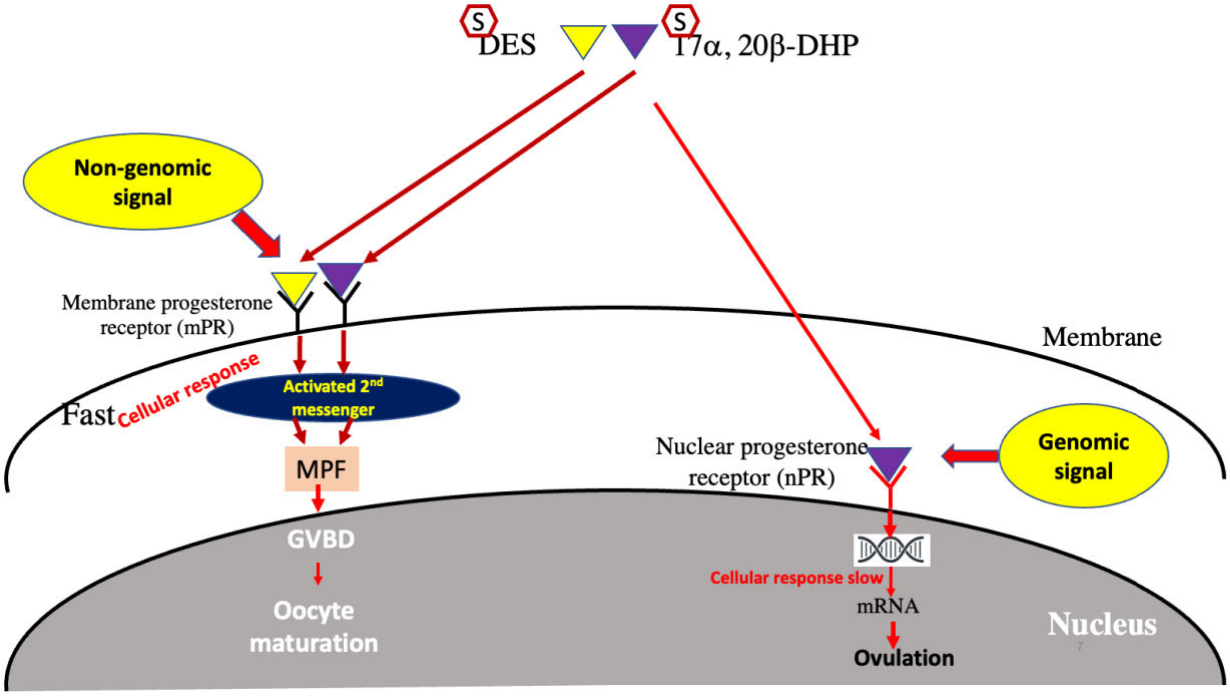


Figure 6: Genomic and non-genomic signal pathway. This model explaining the ligand and receptor interaction and passing process of different cellular signal through the channel. Oocyte maturation and ovulation happened by the action of different steroidal compound where mPR and nPR paly their individual role according to the structural characteristics of the ligand

ABSTRACT

Marine algal flora significantly contribute in the productivity, flow of energy in the ecosystem, remediation of contaminants and nutrient cycling between terrestrial and aquatic ecosystems. Moreover, marine algae also release some chemical, which has some hormonal like activity. As we know that progestins are the synthetic forms of the hormone progesterone, which occurs naturally in the body and acts a very significant role in controlling reproduction. The physiological effects of progesterone are mediated through modulating the expression of genes linked with nucleus progesterone receptors (nPRs), but the discovery of membrane progestin receptors (mPRs) brought new insights into progesterone action. Current study attempted to cultivate the marine algae known as "*Padina*" in *in-vitro* condition to extract the natural active compound. Firstly, the species of the *Padina* was identified by DNA sequencing. The Molecular analyses using mitochondrial *cox3* genes as molecular markers confirmed the species as *Padina arborescens*. The concentrated samples were separated through high performance liquid chromatography (HPLC). Several steps of HPLC analysis were accomplished to obtain the final refined samples from two chromatographic peak. The binding affinity of the samples were significantly potent towards the mPRα, which was governed by the steroid binding assay with the crushed cells membrane of transfected cell comprising the mPRα gene. Subsequently, the existence of two specific chemical compound in the samples were unveiled by ESI mass spectrometry. After the spectrophotometric analysis that the purified compounds were fluorogenic substances. Meanwhile, the purified chemical exhibited the antagonistic attributes with 17,20β-DHP in order to induce the zebrafish oocyte maturation and ovulation *in-vivo*. Based on the current findings, it can be concluded that the compound from the marine padina has huge binding affinity towards mPRα as natural hormonal compound as well as the inhibitory effects of zebrafish oocyte maturation and inhibition. However, determination of the chemical structure of the compound would be our future prospect as new pharmaceutical candidate as well as to make the compound commercially available.

INTRODUCTION:

Hormones are highly strong chemical molecules produced into the bloodstream by the endocrine gland. In particular, hormones act as a chemical messenger to control and regulate several signaling pathways required for maintaining body function (Ranabir and Reetu 2011). Reproductive hormones play vital roles in the reproductive systems of both male and female puberty development, as well as their growth and sexual character. The reproductive hormones are secreted by the anterior pituitary gland as well as the gonads. These reproductive hormones include both steroid and peptide hormones (Manocha *et al.* 2018). Different receptors aid these reproductive hormones to alter the transcriptional activation of target genes (Fuentes and Silveyra 2019). The major gonadal hormones include Estrogen, Progesterone, Testosterone, placental hormone, Human Chorionic Gonadotropin, Anti-Mullerian Hormone (Barton *et al.* 2018).

Latterly, scientists are trying to be unveiling new hormonal compound from marine organisms like seaweeds, sponges, algae, fungi or corals to understand their potential biological activities (Blunt *et al.* 2007, Yegdaneh *et al.* 2013, Kuda *et al.* 2002, Lafarga *et al.* 2020). Though, seaweeds and marine algae-derived bioactive compounds have already been considerate as potential pharmaceutical agents due to the present of varieties effectivity including the antifungal, antibacterial, antiviral, antioxidant or cytotoxic activities and food sources like acting as (TÜney *et al.* 2006, Ghannadi *et al.* 2012, Mehdinezhad *et al.* 2016, Trono 1997, Vaseghia *et al.* 2019). Therefore, discovering and examining the activities of these marine algae will provide new sight of determining the pathological, pharmacological, and hormonal activities, which will be useful in overcoming clinical development obstacles (Manocha *et al.* 2018).

However, among all the hormones, steroids like progestins are key regulators of controlling reproduction. Even the process of ovulation and maturation of fish oocytes are induced by progestins through nongenomic and genomic actions (Rezanujjaman *et al.* 2020). Progestins are the synthetic forms of the hormone progesterone, which occurs naturally in the body and acts a very significant role in controlling reproduction (Tokumoto *et al.* 2017). The expression of different genes associated with progesterones are controlled by different receptors like nuclear progesterone receptors (nPRs) and membrane progestin receptors (mPRs) (Tokumoto *et al.* 2017) 12). Indeed, the mPRs are 7-transmembrane proteins that contain the PAQR family's seventh

gene, including mPR (PAQR7), which is one of the protein subtypes implicated in physiological functions in reproductive tissues (Thomas and Pang 2013).

The mPRs act as a novel target for endocrine-disrupting chemicals (EDCs). Some natural or synthetic hormone substances or chemicals that interact with mPRs are presently being investigated as potential new medicines or EDCs (Diamanti-Kandarakis *et al.* 2009). Previous studies explained that the oocyte maturation in fish induced by progestin induction was examined to understand the nongenomic steroid actions causing endocrine disruptions. Some EDCs such as diethylstilbestrol (DES) analogs showed similar induction activity of meiotic maturation in goldfish and zebrafish oocytes (Tokumoto *et al.* 2006). To understand the mechanistic pathway of EDCs of interacting outer membrane receptors (OM) mediated by progestins via receptor binding assays was observed in the plasma membrane of goldfish ovaries and breast cancer cells transfected with goldfish membrane progestin receptor (mPR). As a result, high affinity, saturable and single binding sites for goldfish maturation inducing steroid (17,20β-DHP) was found in OM of both ovaries and mPR transfected cells (Tokumoto *et al.* 2006). The interaction between mPR and DES or DES analogs, as well as their OM binding activities, explained the endocrine disruption caused by the binding, as well as replicating the activity of nongenomic progestins (Tokumoto *et al.* 2006).

Meanwhile, *Padina* is another experimental algae which is now being targeted to identify similar mPR binding activities or to find out whether the *Padina* is able to secret steroid-like compounds or not.

Some studies have already proved that different species of *Padina* have multiple cytotoxic activities like *Padina australis* has anti-tuberculosis activity, anti-proliferative activities of *Padina sanctae-crucis*, presence of phytosterol in *Padina gymnospora*, *P.pavonica* & *P. spliquosa*, presence of polyphenols in *P. boergesenii*, etc (Peter *et al.* 2013, Hakim and Patel 2020, Vaseghia *et al.* 2019). Considering the previous research on nongenomic actions of progestins and interaction between mPR and DES, our current research focussed mainly on determining the steroid-binding ability of chemical discharge from *Padina* by detecting with mPR cell. For this purpose, we tried to follow several purification and characterization methodologies such as novel High-performance liquid chromatography (HPLC) and analytical techniques such as NMR spectroscopy (1H and 13C NMR) for qualitative and quantitative

analysis and mass spectrometry (ESI-MS) to gain further insight into the complexity of these bioactive compounds.

Study Rationale and hypothesis

Scientists are now extremely concerned about identifying the new mPR ligand. Previously, one of the research projects was conducted by our laboratory group on detecting the membrane progestin receptor (mPR) interacting compound of coral sea water where they collected samples from the Mauritius. The samples were collected from three different area (shore, lagoon and reef) in the sea (Fig. 7A).

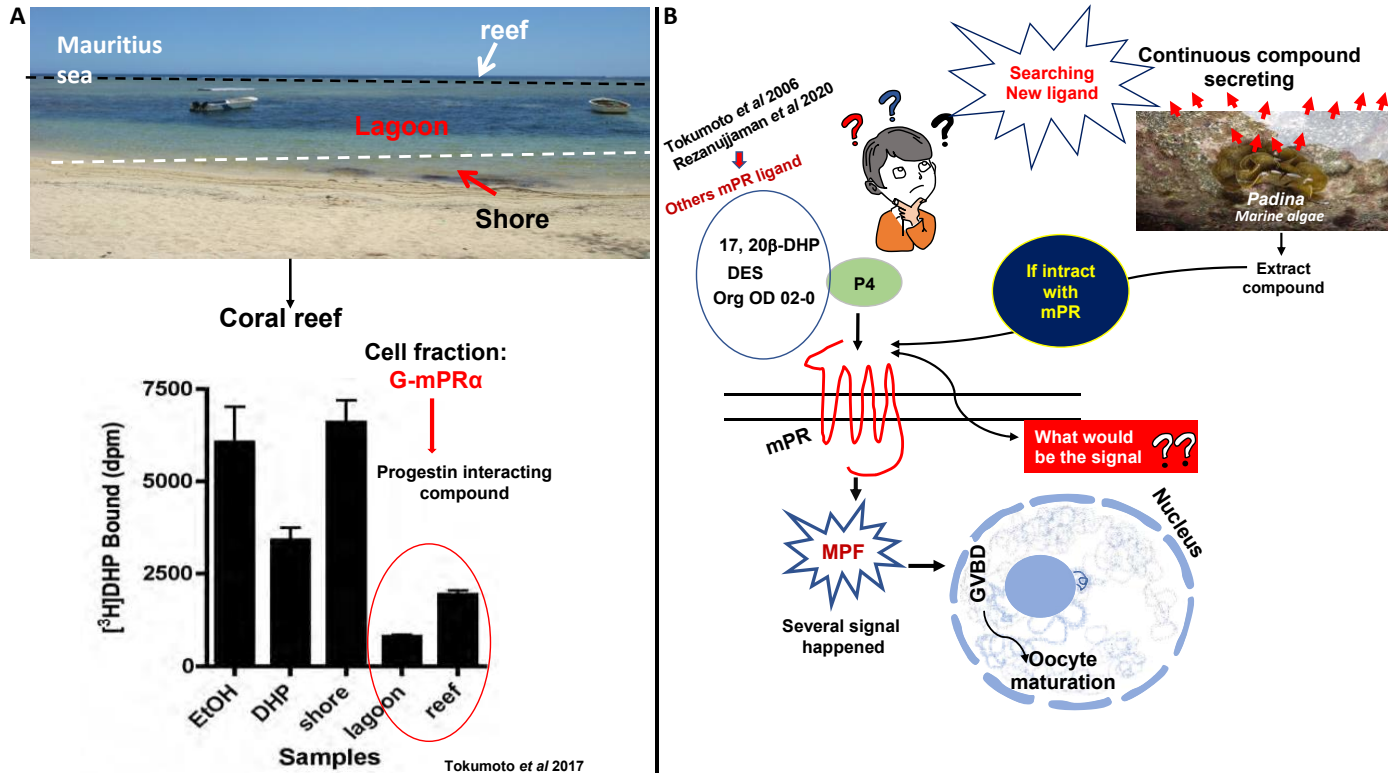


Figure 7: Motivation of the study from coral reef releasing mPR interacting compound panel A. Panel B) the study hypothesis; after interaction of DHP, DES Org OD 02-0 with mPR, searching the new compound from marine algae *Padina* has been demonstrated.

The reef was considered the point closest to the edge of the lagoon, the lagoon was the central point between the reef and the beach, and the shore was in the area closest to the beach. After processing the samples, the potent binding was found in the sample from lagoon and reef with the mPR (Tokumoto *et al.* 2017). Some other mPR ligand as DES, DHP, and Org OD 02-0 have already been established in early research after discovery of membrane progestin receptors (mPRs) as a nongenomic signalling pathway (Nagahama & Adachi, 1985, Zhu *et al.* 2003a; Zhu *et al.* 2003b, Rezanujjaman *et al.* 2020) (Fig. 7B).

Based on the early published findings, it is reasonable to hypothesize that such artificial or natural compounds, rather than steroid hormones, have the ability to act as mPR ligands. To aid in the search for novel pharmaceuticals and EDCs, screening systems for mPR-interacting chemicals must be established.

Aims and objective of the study

Current study selected on of the very potential marine algae *Padina* to determine the new mPR interacting compound which will put a noteworthy contribution in hormone study or reproductive biology. The purpose was given bellow:

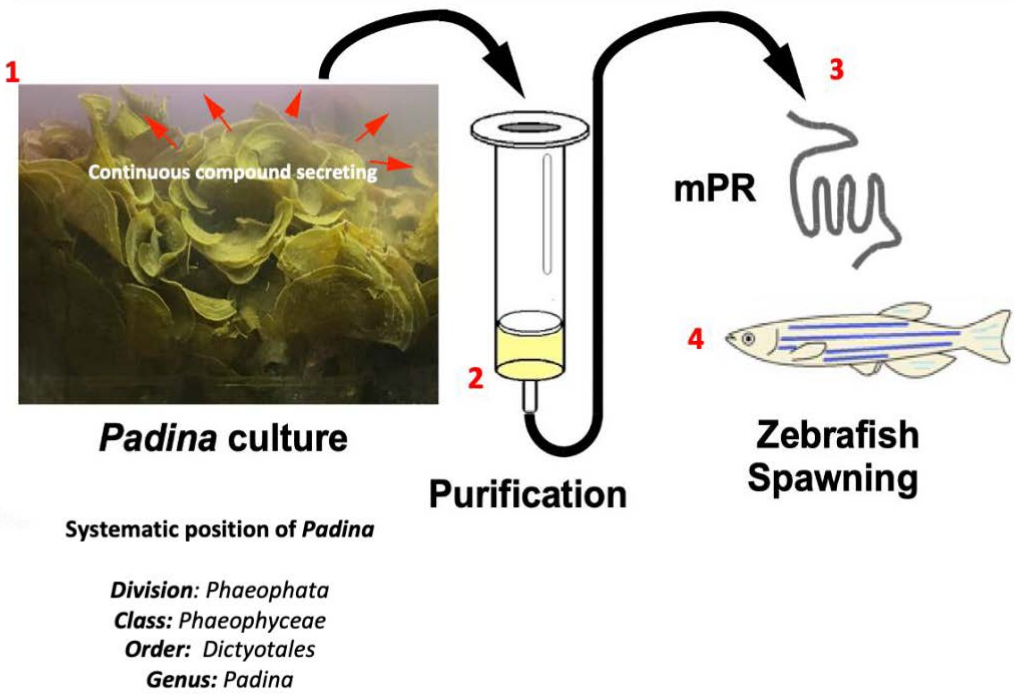


Figure 8: The graphical expression of the aims of our study:

1. The *Padina* spp. were collected and planted in the artificial aquarium for their breeding, the compound was accumulated by filtration system as *Padina* secreted continuously in their habitat.

2. after collection of compounds, the purified fractions were collected through high-performance liquid chromatography (HPLC).

3. The mPR interacting activity of the compound was confirmed by radiolabeled binding assay

4. Finally, the physiological activity was revealed by *in vitro* and *in vivo* oocyte maturation and ovulation-inducing assay by means of zebrafish

MATERIALS AND METHODS

Sampling location

Several hundreds of brunches of marine algae, Padina arborescens, were collected at marine field of Shimoda Marine Research Center, University of Tsukuba (34º66'64.87"N, 138º93'89.05"E) or Mochimune marine filed of Faculty of Agriculture Regional Field Science Education and Research Center, Shizuoka University (34º92'07.51"N, 138º36'82.14"E) in the Shizuoka Prefecture, Japan (Fig. 9) All the samples were transported in the laboratory by portable cooler box.

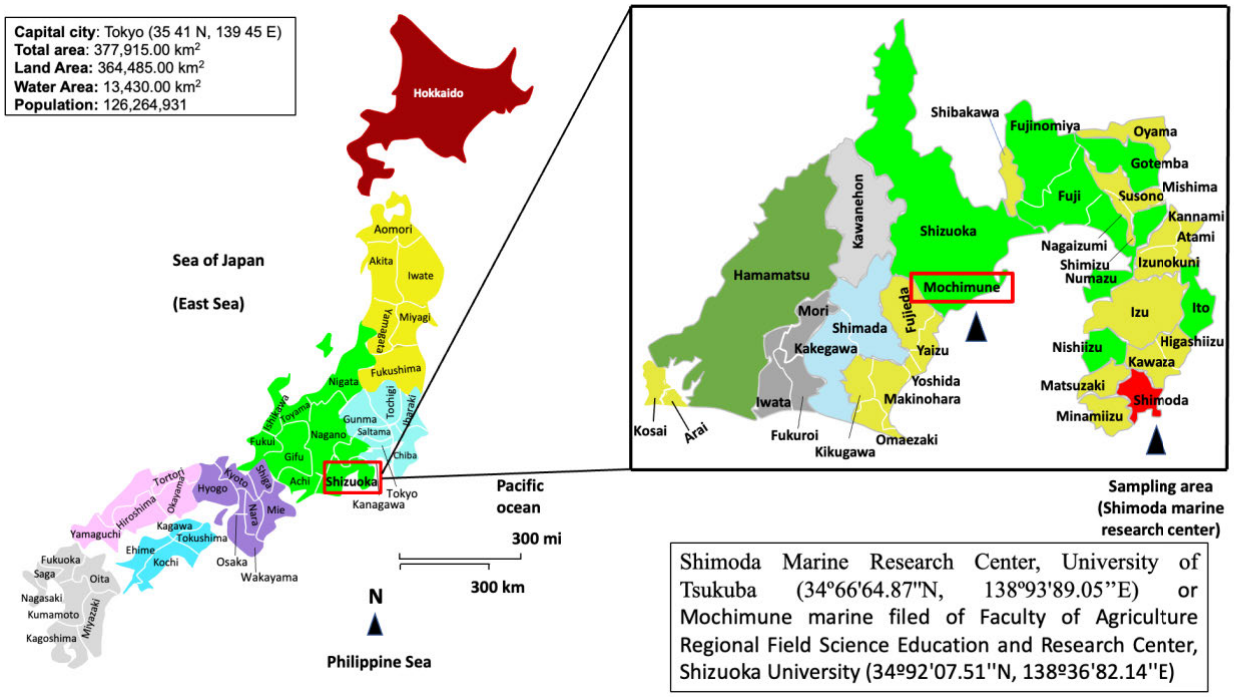


Figure 9: Indication of sampling location. This image showing the whole Japan map and the map of Shizuoka city. In the right-side map is the city of Shizuoka where black arrow head indicating the two sampling place.

In-vitro plantation of *Padina*

All the cultivated *padina* were planted in the *in-vitro* condition (aquarium) which was prepared at Mochimune. Before collecting the samples, the 120cm long aquarium tank was prepared with seawater and small size gravel stone. The LED light was introduced for the photosynthesis of the marine algae *Padina* (Fig. 10).

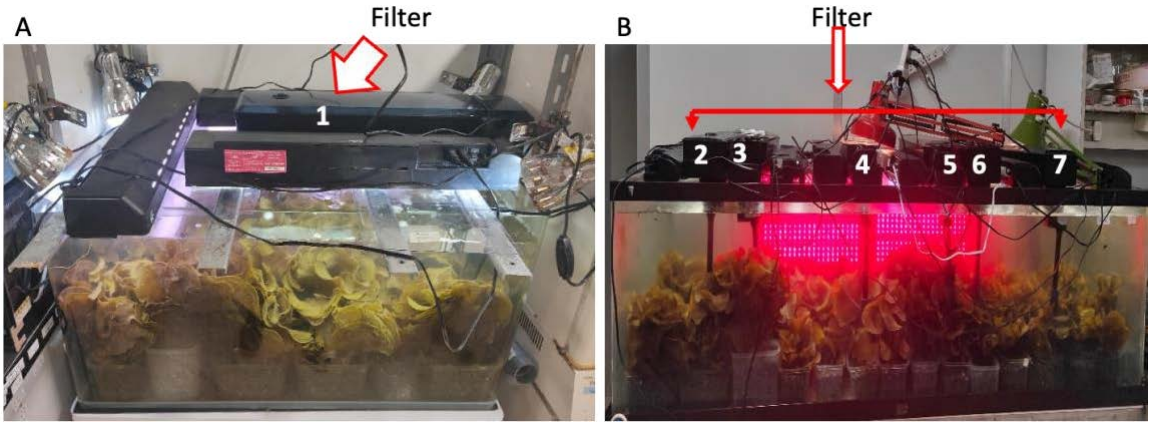


Figure 10: *Padina* plantation and collection of compounds in aquarium with possible all the ambient environments. Panel A) and Panel B) showing the habitat where gravel stone and LED light were set for the survival of *Padina*. Finally, filter was set to collect the compound

Identification of the *Padina* species

To identify the species of the *Padina*, the genomic DNA was extracted through the NucleoBond HMW DNA kit (REF 740160.20, LOT 2104/002, MACHEREY-NAGEL Gmbh & Co. KG, Germany). Prior to extract the DNA, the leaf portion of the collected *Padina* was vigorously washed with citric acid solution and dried the leaves properly. Afterward, the dried leaf of the *Padina was* grinded by Ball Mill machine (PM 100). Gel electrophoresis (1% agarose gel with marker 19) was performed getting the DNA pattern. DNA sample was stored at 4^{o} C in the refrigerator for further amplification by polymerase chain reaction (PCR).

PCR was carried out, using the purified DNA as a template, with a TaKaRa PCR Thermal Cycler Dice^{R} Touch (Takara Bio. Inc., Shiga, Japan)) Reaction Kit. The *Padina* sequence was amplified by the forward and reverse primer *cox3*. For cox3 PCR the forward and reverse primer were, respectively, *cox3*-F (5′-TGGTAACTTGGTGGCGTGATA-3′) and *cox3-R* (5′-GGTGCTGTCGGCTAAAATGG-3′). To determine the optimal annealing temperature, gradient PCR was implemented ranging from 55-65^{o} C. The *cox3* F1R1 amplified successfully. The PCR conditions for *cox*3 F1R1was as follows: an initial denaturation step at 95°C for 5 min, followed by 95°C for 0.5 min, annealing at 55°C for 0.5 min, extension at 72°C for 0.5 min for 35 cycles, and final extension at 72°C for 10 min. PCR products were checked for length and yield by electrophoresis on 1.5% agarose gels dyed with Midori Green Advance DNA stain (Nippon Genetics Europe, Dueren, Germany) (Ni-Ni-Win *et al.* 2008).

Sample preparation for sequencing from PCR products:

The primer contents were removed from the PCR products by alkaline phosphatase treatment. Master mixture was prepared, the 25µl PCR products. A total of 15µl samples were prepared by using 10µl PCR products and 5µl from master mixture and incubated in PCR machine (for cycle 1, 30° C for 15min and 80° C for 15min). The concentration of the PCR product was measured by taking the absorbance. After getting the maximum concentration 10-40ng/2µl in 30 times diluted samples, a total of 14µl final samples were prepared by using11.3µl DDW, 0.6µl F primer and 2µl purified PCR product for sequencing.

Compound collection and column application

Several fish cultivation filters were subjected to prepare and set to accumulate the secreted compound from *padina*. After 15-20 days later, all the filters were detached and washed vigorously with double distilled water (DDW) to remove the seawater especially the salt content. Afterward, each of the filters was saturated with absolute ethanol (1 liter for one filter) to extract the trapped particle/ compound (Tokumoto *et al.* 2017). The whole samples were diluted 10 times with DDW and applied in the ODS column (50µm, 1.8×11.4 cm, 7g, Yamazen corporation, Japan) connected to the peristaltic pump which traps particle >1 µm. Approximately 10-20 L of the sample was throughout into a single column (Fig. 11).

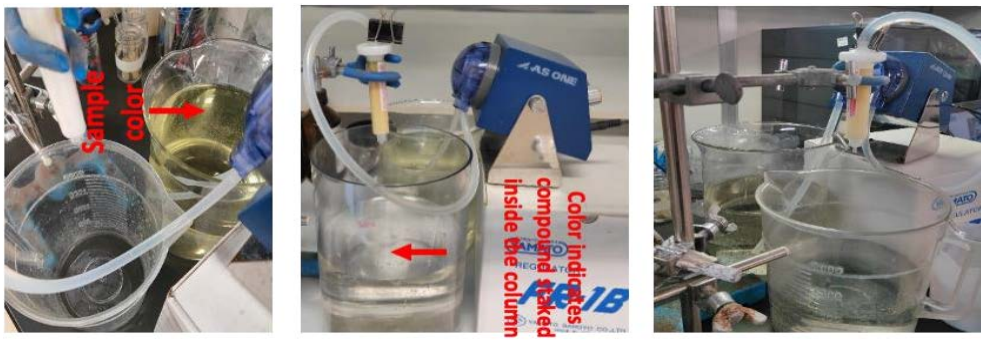


Figure 11: Application of samples on column for HPLC. Collected samples were applied on the ODS column by peristaltic pump, in the picture the original color of the samples were yellow but after application on column the throughout samples color were colorless.

Eluting the compounds from the column (HPLC analysis)

HPLC is a reliable analytical technique for determining the qualitative composition of non-volatile chemicals such phenolics, terpenoids, and alkaloids. (Bonta 2017). It is very efficient,

allowing for faster and more accurate analytical separation as well as a higher sample loading capacity (Chen *et al.* 2012, Long *et al.* 2014). The column is an important part of HPLC. The particles employed as the stationary phase are contained in the column. The stationary phases are commonly packed in a column with a 2–5 mm diameter size and have particle sizes ranging from 3 to 50 m. Reversed-phase (RP) columns are used for the majority of separations (Oniszczuck and Hawryl 2011). In the present study, we applied water (solvent A) and the very strong organic solvent acetonitrile as solvent B along with the small amount (0.05% for 1L) of tri-fluotoaceticacid (TFA) to separate the concentrated compound into the column (Fig. 12). ODS-SM (1.8×11.4 cm) and silica-based C18 columns (4.6×30 cm) containing octadecyl silica group or C18 groups (Tosoh corporation, Shiba-Koen first Bldg. Minato-ku, Tokyo) were used in the first and second step separation respectively. Finally, the Phenyl column (Tosoh corporation, Shiba-Koen first Bldg. Minato-ku, Tokyo) was introduced for final separation which contains strong pi-pi interaction. The UV wave was 254nm at 40^{o} C for each of the column applications. The HPLC condition was conceded as gradient elution mode.

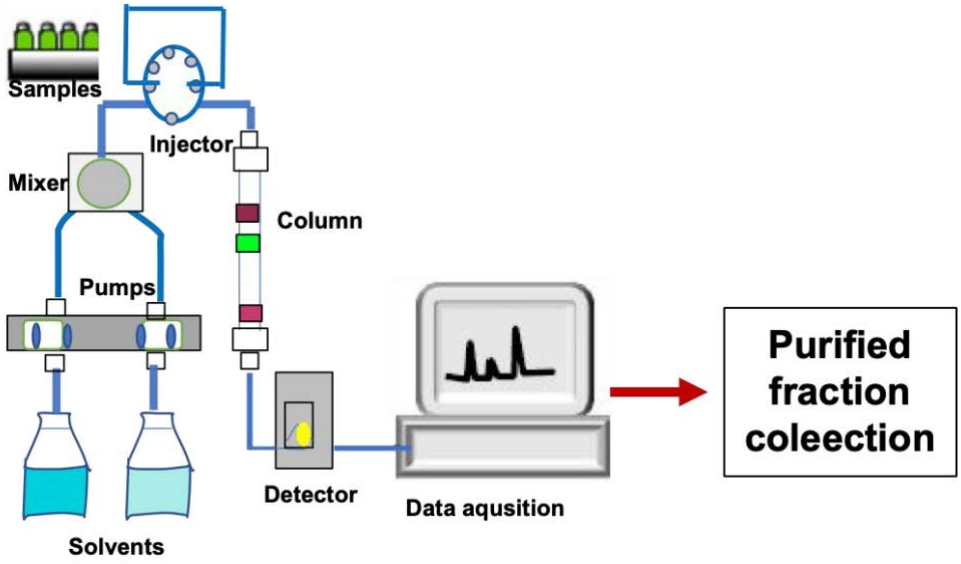


Figure 12: A simple illustration of HPLC machine. How the two solvent elute the compound and the chromatogram peak observed in the computer monitor through detector.

The initial conditions were 0.0% solvent B by 10min at flow rate of 0.5ml/min for the ODS-SM column. The gradient program elevated to 100% solvent B by 90min to 100min. In the case of C18 column, firstly the column was equilibrated with solvent A before injecting the samples and set the program as 0.0% solvent B by 10min at flow rate 2ml/min and the program lifted to 100% by 60min to 80min. Meanwhile, the initial conditions for phenyl column were 0.0% solvent B by 10min at flow rate of 2ml/min which was further raised to 100% solvent B by 90min to 100min. The samples were injected after equilibration of the column with solvent

A. According to the chromatographic peak after the first step of HPLC, the corresponding fractions were collected in the sterile test tube (Kaweewan et al 2020).

Lyophilization of the collected fractions

The collected fractions were lyophilized for removing the solvents from a sample via sublimation, which was achieved with a low vacuum that boils water below its freezing point. After several hours of lyophilization, the dried samples were collected and dissolved with ethanol for further purification. The extracts were repeatedly subjected to HPLC purification and lyophilization to obtain the refining compound (Franks 1998, Li *et al.* 2005).

Radiolabeled ligand binding assays

Membrane fraction preparation:

The previously cultured cells were subjected to wash three times with PBS. The washed cells were dragged out aseptically into HAED buffer solution (Hepes, 25 mM; NaCl, 10 mM; EDTA 1 mM; dithiothreitol, 1 mM; pH 7.6 at 4 ºC), and the collected cells were sonicated for 15 sec to disrupt cell. After sonication the cells were centrifuged at 1,000 × g for 7 minutes to remove any nuclear and heavy mitochondrial material. To obtain the plasma membrane fraction the resulting supernatant was further centrifuged at 20,000 × g for 20 minutes (Fig. 13) (Tokumoto *et al.* 2007).

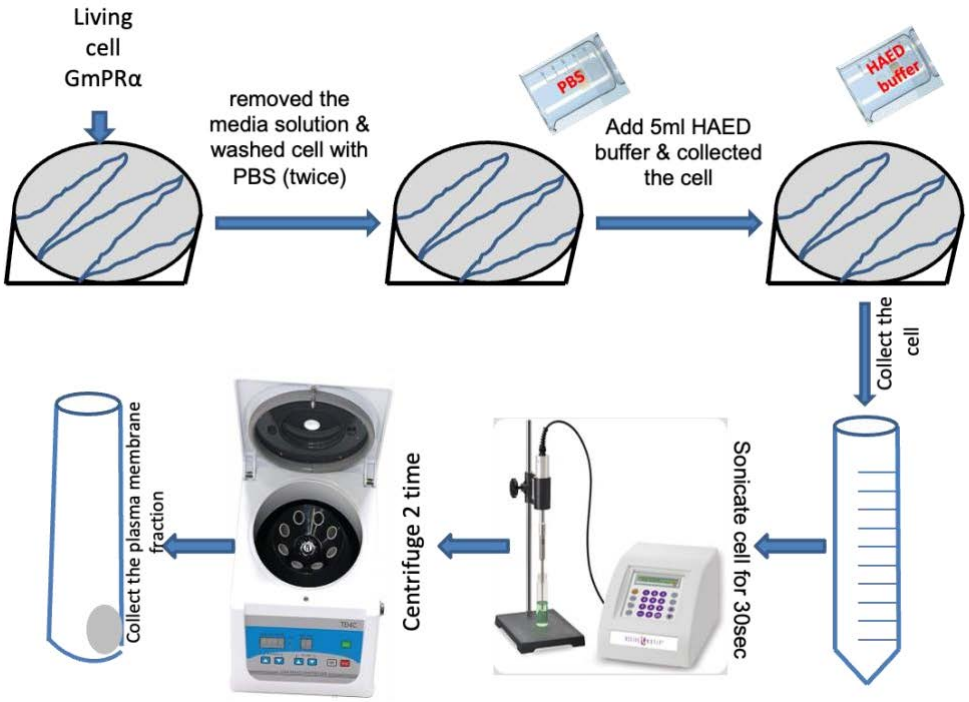


Figure 13: Membrane fraction preparation protocol. Firstly, removed the media solution nd washed the cell with PBS two times then the mPR cells were collected by using 5ml HAED. The cell was taken in the falcon tube and sonicated for 30sec to disrupt the cells. Final plasma fraction was collected after two times of centrifugation.

Membrane binding assays

Using radio-labeled progestin, steroid binding tests were used to evaluate the mPR-interacting activity of compounds in samples. [1,2,6,7 3H]-17α-Hydroxyprogesterone (85 Ci/mmol) was purchased from Amersham Biosciences (Piscataway, NJ, USA) and enzymatically converted to radiolabeled 17,20β-DHP by 3α,20β-hydroxy-steroid dehydrogenase (Sigma, St Louis, MO, USA), as described by Scott et al. (1982). Following previously known techniques, progestin receptor binding was evaluated in the membrane fraction. (Tokumoto *et al.* 2007). Four µL of ethanol or samples dissolved in ethanol was added into each tube. Two hundred µL of membrane fraction and 200 µL of [^{3}H]-17,20β-DHP solution were added and incubation was started. A set of three tubes contained 2 nM [^{3}H]-17,20β-DHP (total binding), and another set of three tubes contained samples to measure competitive binding activity. After 30min of incubation at 4°C, the reaction mixture was filtered through GF/B filters (Whatman, Maidstone, UK) those were previously soaked in HAED buffer containing 2.5% Tween 80. After filtration, all the filters were rinsed three times with 5 mL of washing buffer (25 mM HEPES, 10 mM NaCl, and 1 mM EDTA; pH 7.4 at 4°C). The filters were taken in the scintillation vial trembled properly for 15min through the vial mixer Vix-100 machine. Afterward, the bound radioactivity was measured with a scintillation counter. If the samples contain compounds with the compatible binding site with the progestin binding site of mPRα, then the radiolabeled 17,20β-DHP was displaced by the compounds. The radiolabeled 17,20β-DHP displacement was calculated as a percentage of the maximal specific binding of 17,20β-DHP to the membrane fractions (Fig. 14). (Tokumoto *et al.* 2007).

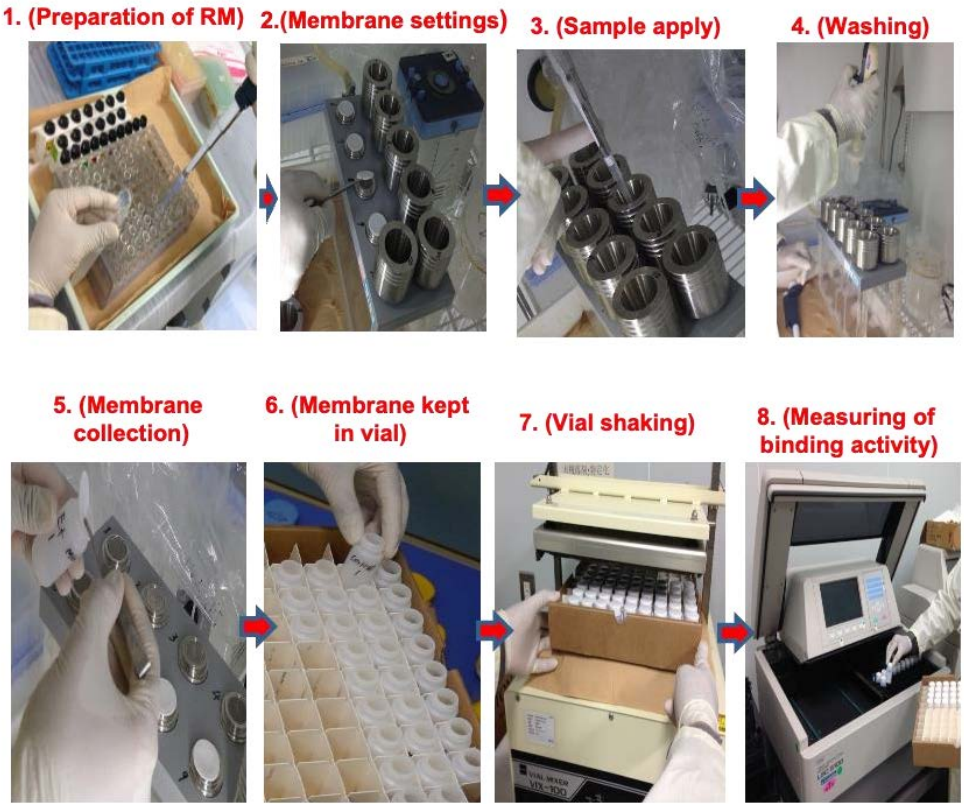


Figure 14: Radiolabeled binding assay protocol. 1) reaction mixture was prepared by using membrane fraction, cold hormone and hot hormone, 2) Membrane was set in the filter cube those were socked in the Tween80, 3) the reaction mixtures were applied onto the filter very gently, 4) added the 5ml of HEPES/HAEW buffer 3 time for each of the cube to wash the unbound molecules 5) membranes were collected by sterile forceps in the vial 6) 3ml of cocktail solution was in the membrane containing vial, 7) vials were shacked properly in Vix-100 machine 8) bound radioactivity was measured with a scintillation counter

Electrospray ionization mass spectrometry (ESI-MS) Analysis

ESI methodology can be used in either positive (ES+) or negative ion mode (ES-), depending on the proton affinity of the compound. MS analysis was performed using JEOL JMS-T100LP mass spectrometer. For accurate MS analysis, reserpine was used as an initial standard (Kaweewan *et al.* 2020).

Determination of Biological Activity of Compound collected from *Padina* on Zebrafish

Zebrafish cultivation process

To conduct the *in-vivo* oocyte maturation and ovulation test, the zebrafish were utilized as model organisms which was previously maintained in the laboratory fish nurturing system with suitable water flow at 28.5°C under the 14 h light/10 h dark cycle. The ethical approval of using

the experimental organisms were permitted (approval no. 2019F-5) by the Institutional Ethics Committee of Shizuoka University, Japan (Rezanuzzaman *et al.* 2020).

In vivo Oocyte Maturation and Ovulation Assay

According to the previously described method, Gravid female zebrafish with full-grown immature oocytes were chosen from a mixed group of 10-50 males and females kept in a 20 cm ×25 cm square acryl case with a 25 cm high continuous flow-through. Females who were proven to be fertile were chosen by pre-spawning with 17,20β-DHP (Tokumoto *et al.* 2011, Tokumoto 2014). Females that had been pre-spawned were held for 8-12 days after spawning until they generated fully mature immature oocytes. On the day of the experiment, females that had not ovulated eggs were chosen by attempting to compress the eggs by squeezing the abdomen of the fish following the onset of light (9 a.m. in our laboratory). Selected females were placed in a glass case with 100 milliliters of water per fish. Agents were administered to the fish in vivo by dissolving each chemical in water at 28.5 ^{o}C (from a 10,000-fold stock in ethanol). After a 4-hour incubation period, the female zebrafish were sacrificed to extract the ovaries. Afterward, the ovaries were plunged in the fresh zebrafish Ringer's solution (116 mM NaCl, 2.9 mM KCl, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.2) containing petri dish. Prior to observe the morphological appearance of oocyte under a binocular microscope (SZX12, Olympus, Japan), the ovaries were manually separated to make those uniform (each carrying 1-10 oocytes) by using fine forceps (Fig. 15A). The oocytes that became translucent were scored to determine germinal vesicle breakdown (GVBD). The presence of a distinct fertilization membrane on the oocytes was used to determine ovulation. In each experimental condition, the rates of GVBD and ovulation were determined in at least twenty oocytes.

In vitro Oocyte Maturation Assay

To determine the physiological effects of the purified compound from *Padina* arborescence on the zebrafish, we flowed the in vitro zebrafish oocyte maturation assay as previously described, (Tokumoto *et al.* 2005). Gravid females with no ovulated eggs were chosen by attempting to squeeze the eggs by pushing their abdomen after the onset of light (9 a.m. in our laboratory). Then the zebrafish ovaries were isolated from sacrificed females and washed in fresh zebrafish Ringer's solution (116 mM NaCl, 2.9 mM KCl, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.2). Using fine forceps, the ovaries were manually dissected into ovarian fragments (each containing more than 20 oocytes). Fully developed immature oocytes were exposed *in vitro* by

incubating them in 4 ml of zebrafish Ringer's solution containing each experimental agent (diluted from a 1000-fold stock in ethanol) at 25.0 ºC or room temperature with gentle agitation (40 rpm). Germinal vesicles (GVs) were examined under a binocular microscope to assess the matu- ration processes (SMZ645, Nikon, Tokyo, Japan). The oocytes that became transparent were scored to determine GVBD (germinal vesicle breakdown). In each experimental condition, the percent GVBD was determined in more than twenty stage 3 oocytes (Fig. 15B).

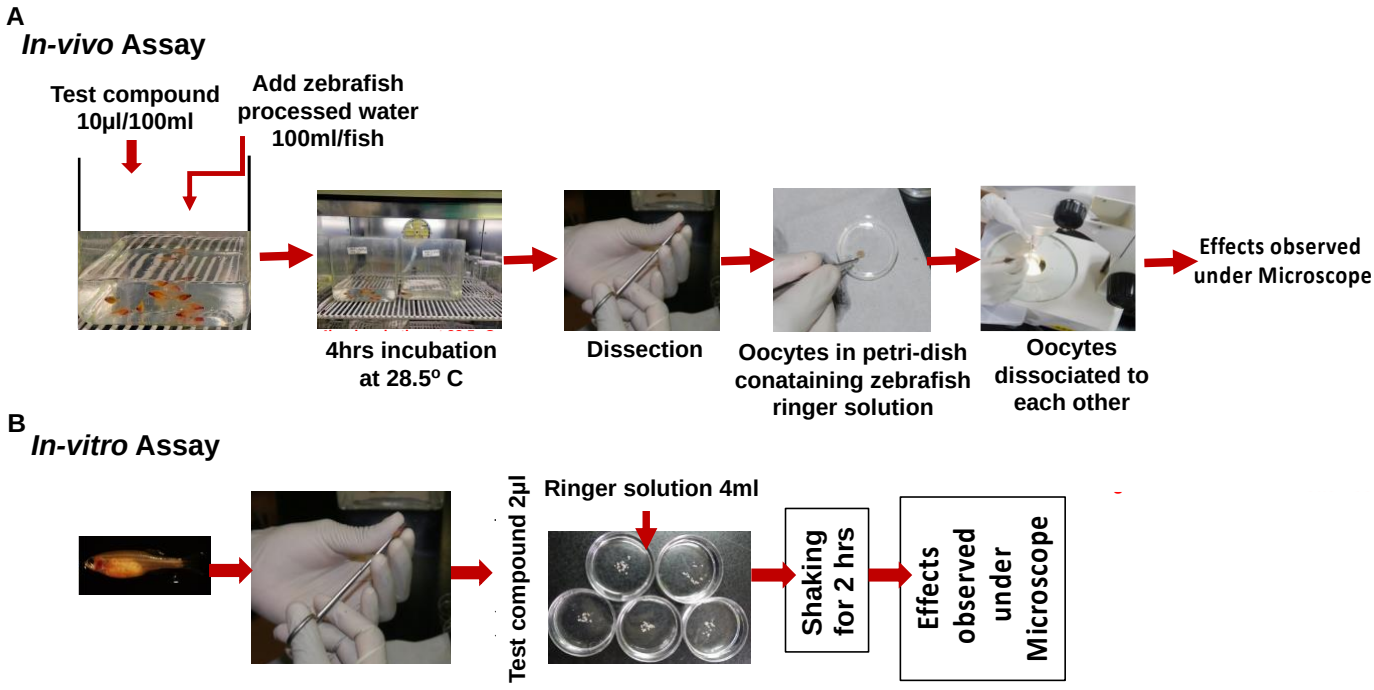


Figure 15: Protocol of *in-vivo* and *in-vitro* assay. Panel A) representing the in-vivo assay where the zebrafish processed water was taken in the tank (100ml/fish) and added the test compound (10µl/100ml water). Then the fish was incubated 4hrs at 28º C. After incubation the fish was dissection, and the oocytes were taken into the Petri dish containing ringer solution. Oocytes were separated to each other by observing under microscope. Finally, the results were observed (either matured or not) under microscope. Panel B) representing the in-vitro assay where the oocytes were collected directly from the fish and taken onto the petri-dish (20 to 30 for one petri-dish) where 4µl ringer solution was added with 2µl test compound. Then the petri-dishes were shacked for 2hrs and observed the results under microscope.

Determination of the action of the test compound on oocytes of the zebrafish

After adding the test compound, the incubation process was started, and the effects was observed. Generally, the immature oocytes were black which has the germinal vesicle and follicle cell indicating as red arrow. After the action of test compound, if the oocytes become matured then it looked like the transparent oocytes and the ovulation showed by the visible fertilization membrane around the oocytes (Fig. 16).

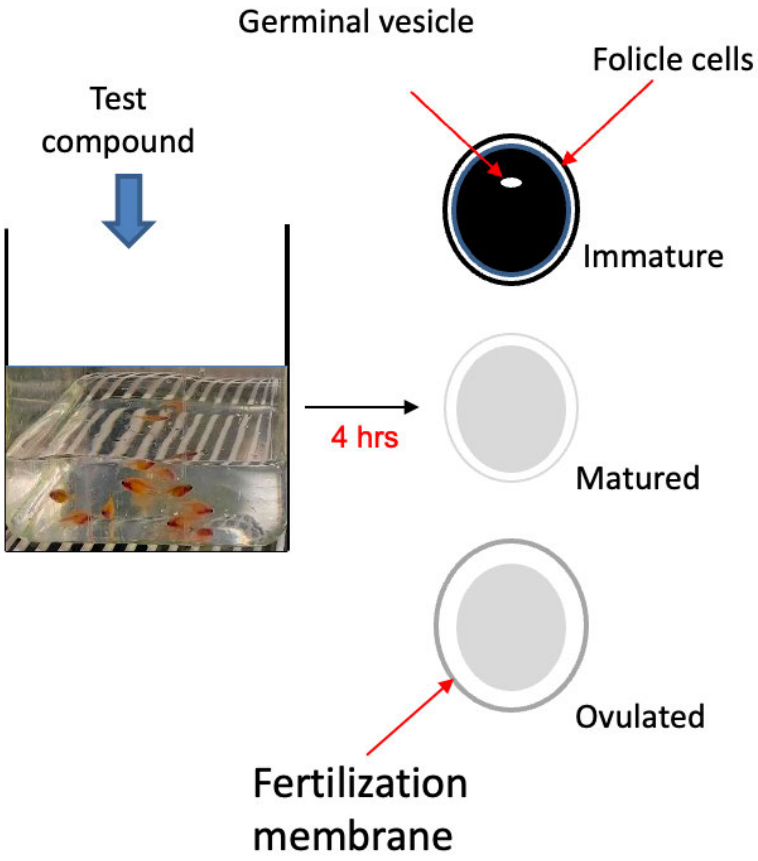


Figure 16: Simple illustration of the stages of oocytes after the action of test compound.

Statistical analysis

All the experiments were performed three times. The GraphPad Prism (San Diego, CA) was customized to calculate the one-way analysis of variance (one-way ANOVA).

RESULTS:

Previously a group of researchers designated a research to detect the mPR interacting chemical from marine organisms (coral reef) from the seawater in Mauritius. They collected the samples from three different areas of coral habitat in the sea such as 1) the reef was considered the point closest to the edge of the lagoon, 2) the lagoon was the central point between the reef and the beach, and 3) the shore was in the area closest to the beach. Among the three, the samples from the lagoon and reef exhibited intense activity with goldfish mPRα (Tokumoto *et al.* 2017). In the present study, we extracted the compound from the marine algae *Padina* by using the fish cultivation filter and introduced different resins to purify the raw compound prior to determine their mPR interacting ability.

Identification of *Padina* species

DNA Extraction of *Padina* sp. and PCR Amplification

The high molecular weight *Padina* DNA was extracted by the NucleoBond HMW DNA kit. The gel electrophoresis results showed the position of the *Padina* DNA corresponding to the marker used (Fig. 17A). The *cox3* PCR primers were successful to amplify and produce PCR product with expected band size 550 bp. The annealing temperature was fixed at 55^{o}C which indicated by arrowhead in the figure 17B. After amplification of the PCR products successfully, the products were cleaned and the NCBI-BLAST tool was used to identify the specimen's sequences according to its species. Our PCR product sequences was found to be almost 100% identical with the sequence of *cox3* gene in the NCBI database. The species identification was revealed that the tested specimen in this study was *Padina arborescens* (Fig. 18).

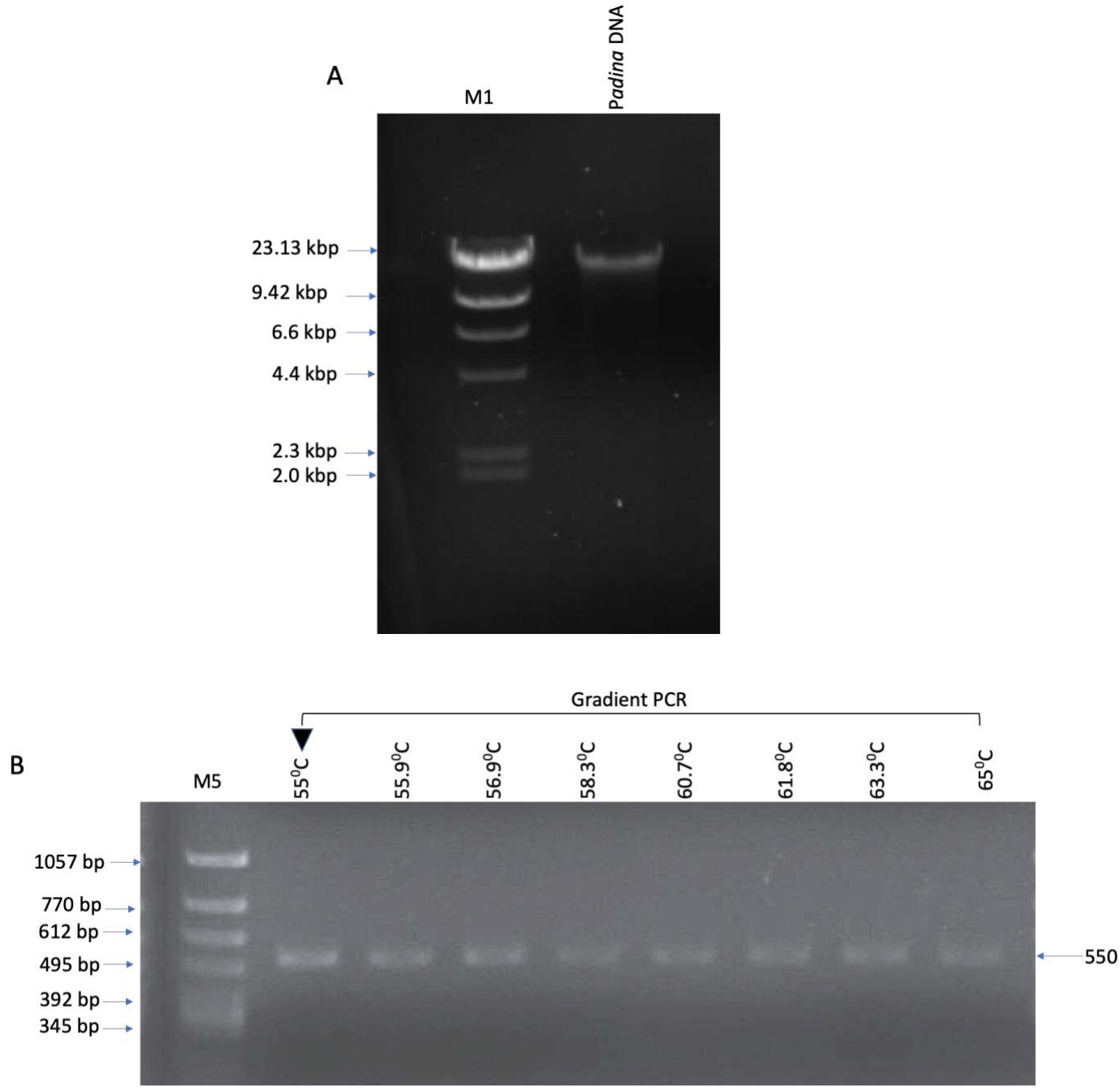
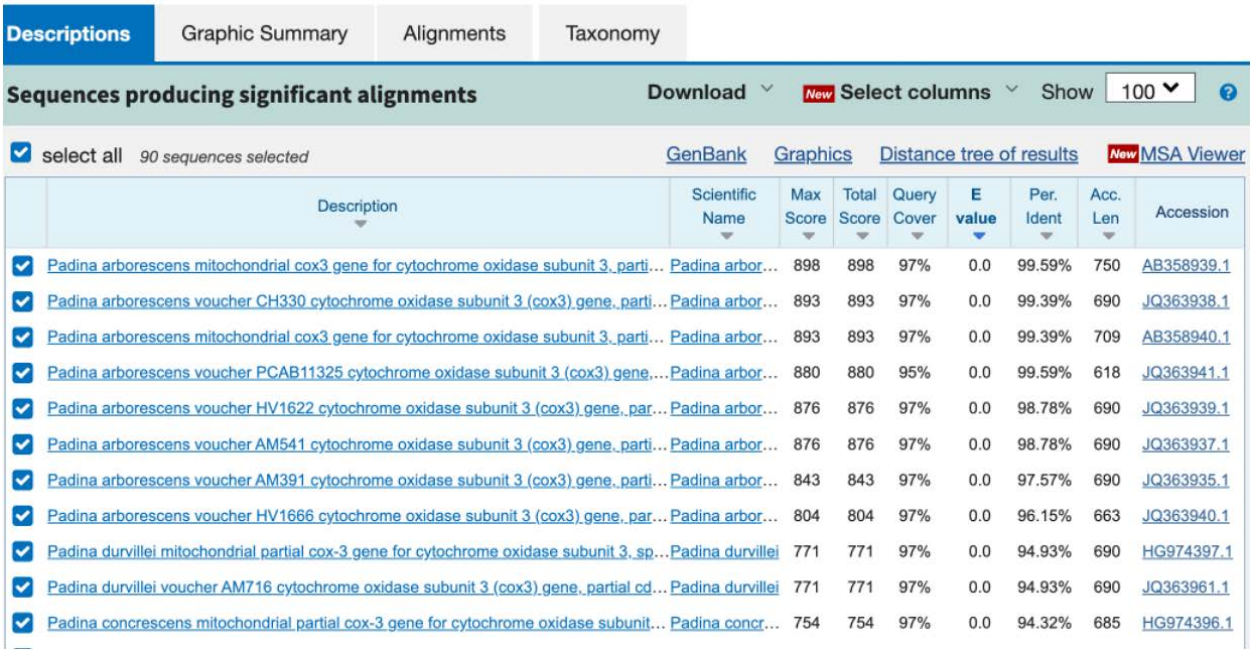


Figure 17: The gel electrophoresis result for the PCR product of *cox3* F1R2 primers.



Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments Download | New Select columns | Show 100

select all *90 sequences selected* GenBank | Graphics | Distance tree of results | New MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Padina arborescens mitochondrial cox3 gene for cytochrome oxidase subunit 3, parti...	Padina arbor...	898	898	97%	0.0	99.59%	750	AB358939.1
Padina arborescens voucher CH330 cytochrome oxidase subunit 3 (cox3) gene, parti...	Padina arbor...	893	893	97%	0.0	99.39%	690	JQ363938.1
Padina arborescens mitochondrial cox3 gene for cytochrome oxidase subunit 3, parti...	Padina arbor...	893	893	97%	0.0	99.39%	709	AB358940.1
Padina arborescens voucher PCAB11325 cytochrome oxidase subunit 3 (cox3) gene,...	Padina arbor...	880	880	95%	0.0	99.59%	618	JQ363941.1
Padina arborescens voucher HV1622 cytochrome oxidase subunit 3 (cox3) gene, par...	Padina arbor...	876	876	97%	0.0	98.78%	690	JQ363939.1
Padina arborescens voucher AM541 cytochrome oxidase subunit 3 (cox3) gene, parti...	Padina arbor...	876	876	97%	0.0	98.78%	690	JQ363937.1
Padina arborescens voucher AM391 cytochrome oxidase subunit 3 (cox3) gene, parti...	Padina arbor...	843	843	97%	0.0	97.57%	690	JQ363935.1
Padina arborescens voucher HV1666 cytochrome oxidase subunit 3 (cox3) gene, par...	Padina arbor...	804	804	97%	0.0	96.15%	663	JQ363940.1
Padina durvillei mitochondrial partial cox-3 gene for cytochrome oxidase subunit 3, sp...	Padina durvillei	771	771	97%	0.0	94.93%	690	HG974397.1
Padina durvillei voucher AM716 cytochrome oxidase subunit 3 (cox3) gene, partial cd...	Padina durvillei	771	771	97%	0.0	94.93%	690	JQ363961.1
Padina concrescens mitochondrial partial cox-3 gene for cytochrome oxidase subunit...	Padina concr...	754	754	97%	0.0	94.32%	685	HG974396.1

Figure 18: Barcode alignment for *Padina* sp. collected form Shimoda and Mouchimune coastal sites of Japan. The best matches for the individual markers are noted with their GenBank accession numbers. The accession numbers are in the right side of the image.

Purification of the compound through HPLC and determination of mPR interacting aptitude through steroid-binding assay

The concentrated samples in ODS-SM column were eluted first by the different concentrations (as mentioned in the methods) of acetonitrile and collected the throughout fraction according to the corresponding chromatographic peak. A total of four peaks were found to be appeared after first step of separation (Fig. 19A). The retention time was estimated within 10-14min for the first peak, 65-68min for the second peak, 80-89min for the third peak, and 90-165min for the fourth peak. Surprisingly, the collected fractions were also separated by different colors as the first peak was colorless, the second peak was partially yellow, the third peak was greenish, and the fourth peak was deep yellow (Fig. 19B). The reactivity of the samples against the mPRα expressing cell was determined through steroid-binding assay. Among the four fractions, the most convincing and consisting binding affinity was evaluated for fraction four in contrast to the binding of the DHP whilst the fractions 1,2 & 3 were unable to bind as a ligand with mPR (Fig. 19C).

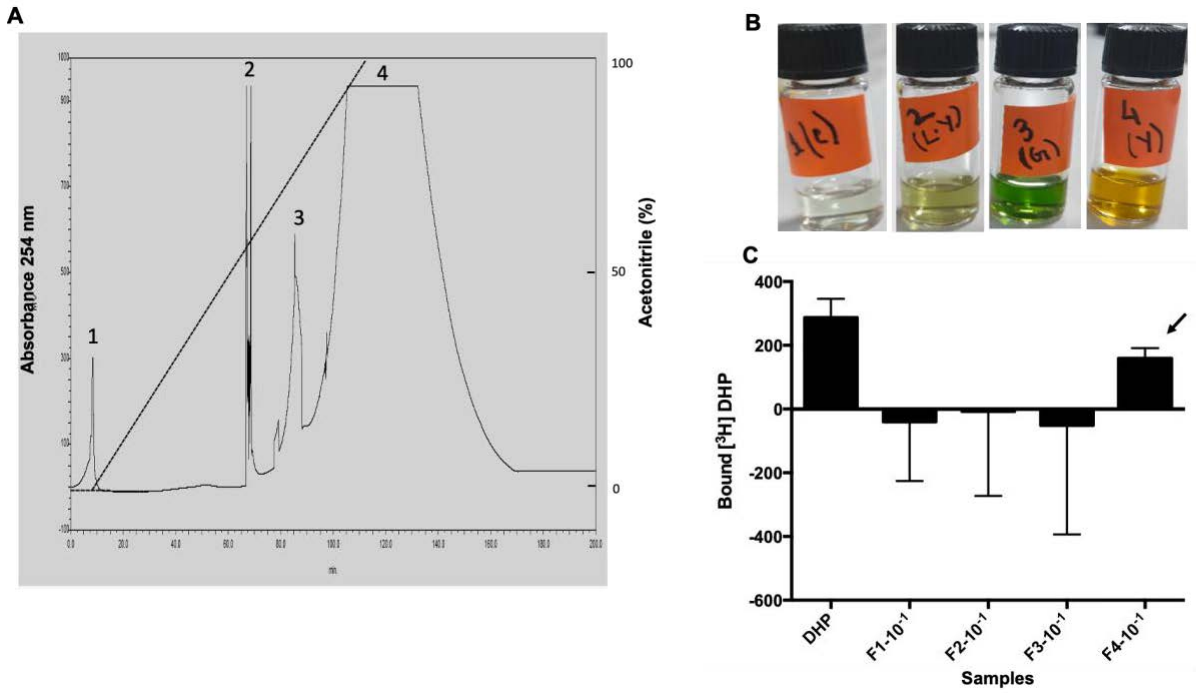


Figure 19: Purification of the compound through HPLC and determination of their mPR interacting ability. Panel A) indicates that total 4 peaks were observed after first step of purification of samples, peak 1 was noticed after 10 min, peak 2 came within 65-68min, peak 3 came with 80-89min and peak 4 was found to be visible within 90-165min. Panel B) indicates the different color of the separated fractions, peak 1 showed no color, peak 2 showed light yellow, peak 3 showed green and peak 4 showed deep yellow color. Panel C) showing the binding activity of the fractions from 4 different peak. According to the graph, only the fraction 4 showed the mPRα interacting. Here, DHP used as a positive control.

Purification of the fraction-4 (bind with mPR) in C18 and determination of their binding activity

After the confirmation of the interacting affinity of the sample from fraction 4, we started to purify the samples through HPLC in C18 and phenyl column. In the case of C18 column, a total of four chromatogram peaks were detected, the first peak was found within the retention time of 42-44min, the retention time of the second peak was 45-48min, the third one came out after 50min and continued up to 55 and the retention time (58-70min) was recorded for the fourth peak (Fig. 20A). The respective fractions according to the chromatogram peaks were collected and lyophilized to make them dry. The dry samples were dissolved in ethanol and performed the steroid-binding assay to determine their activity against mPRα. Among the four

fractions, the samples from fractions 3 & 4 showed the steady binding affinity to mPR in the distinction of DHP while fractions 1 & 2 did not show any activity (Fig. 20B).

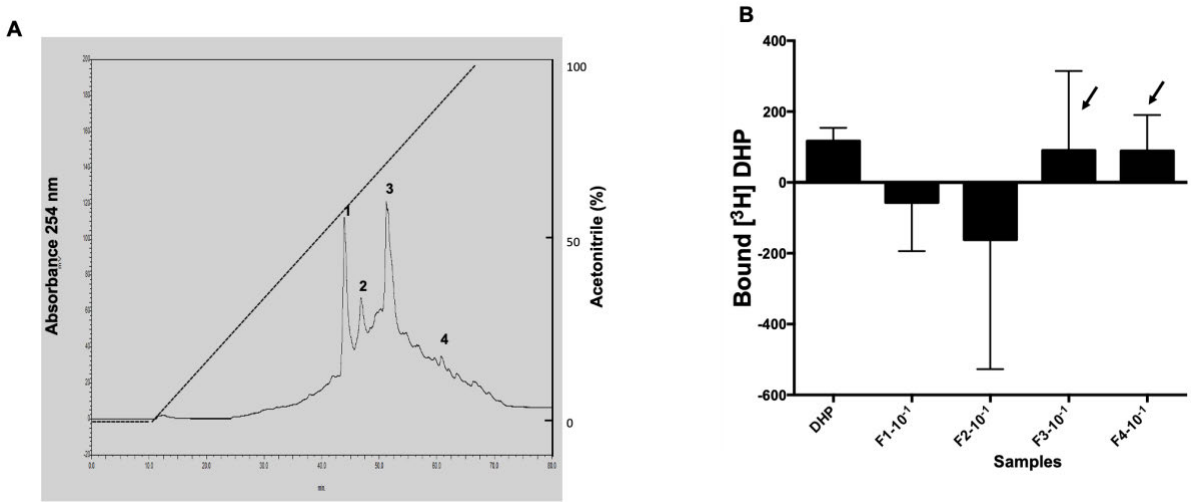


Figure 20: Purification of F4 on C18 column and further evaluation of mPR activity. Panel A) after 2nd step of purification, again 4 peak was found. Panel B) showing the binding activity with mPRα cell. Here, F3 and F4 shoed the binding activity.

Final step of purification on phenyl column

As the final step of purification, the samples from fractions 3 & 4 were subjected to apply on phenyl column. Both of the samples were produced two chromatogram peaks, the retention time of the first peak and the second peak was within 10-16min and 44-60min respectively (Fig. 21A). The binding affinity of both the samples against mPR cells was confirmed by steroid bind assay (Fig. 21B).

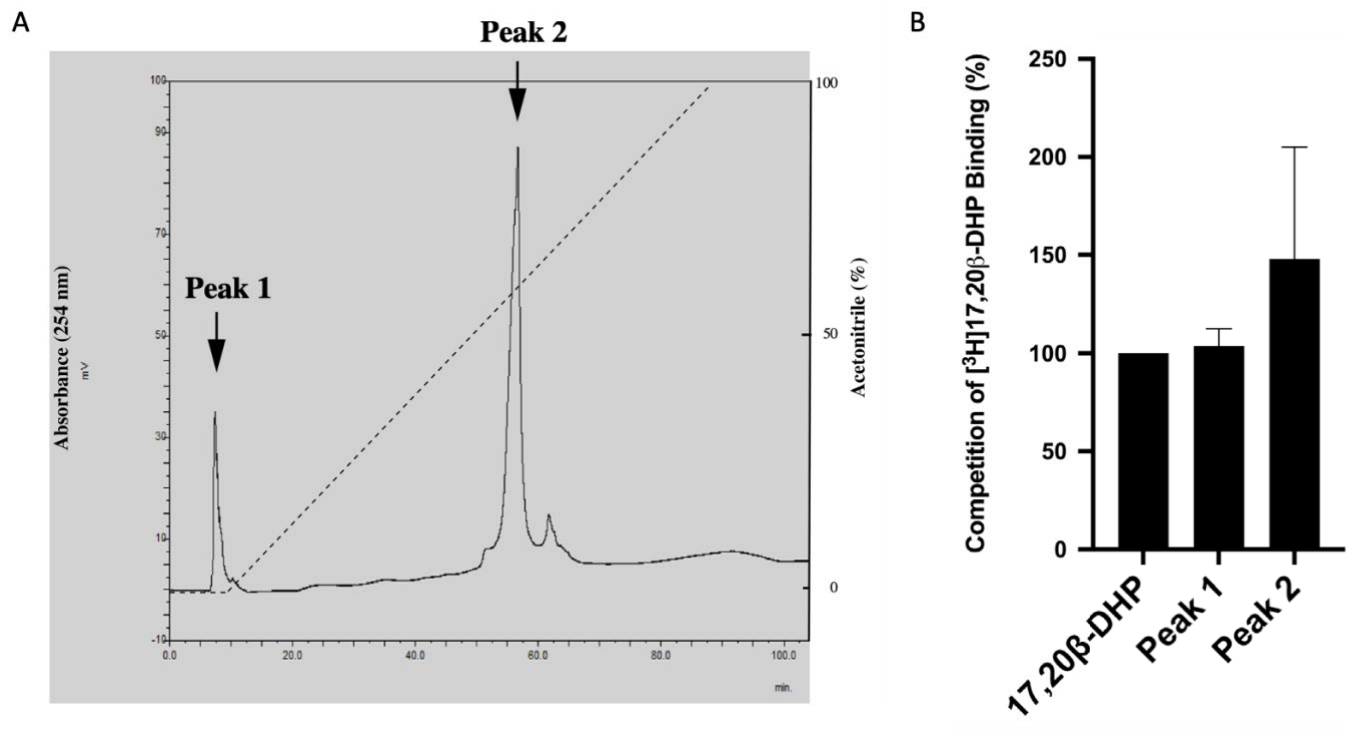


Figure 21: Final step of purification and determination of binding activity. Panel A) chromatogram of separation by TSKgel Phenyl-5PW RP column Fraction with mPRα-interacting activity from ODS column was separated by linear gradient of acetonitrile (0-100%) in acidic condition (0.05% TFA). Peaks in flow-through fractions (Peak 1) and fractions eluted by acetonitrile (Peak 2) were obtained. Panel B) competition by compounds for binding of 17,20β-DHP to mPRα receptors. Samples were incubated with 2 nM [^{3}H]-17,20β-DHP. Competition for [^{3}H]-17,20β-DHP binding expressed as a percentage of maximum specific 17,20β-DHP binding. 17,20β-DHP, 17α, 20β-dihydroxy-4-pregnen-3-one.

Determination of molecular mass of the compound

Electrospray ionization is the most common ionization technique used in chemical and biochemical analysis today. It can investigate the molecular composition of liquid samples when connected to a mass spectrometer. A wide range of chemical substances can be ionized using electrospray (Kaweewan et al 2020).

The major signal was represented by arrowhead in the graph (Fig. 22). Electrospray ionization mass spectrometry (ESI-MS) analysis suggested peak 1 containing the compound with the

molecular mass of 554 Dalton and peak 2 containing the compound with the molecular mass of 554 and 437 Dalton (Fig. 22).

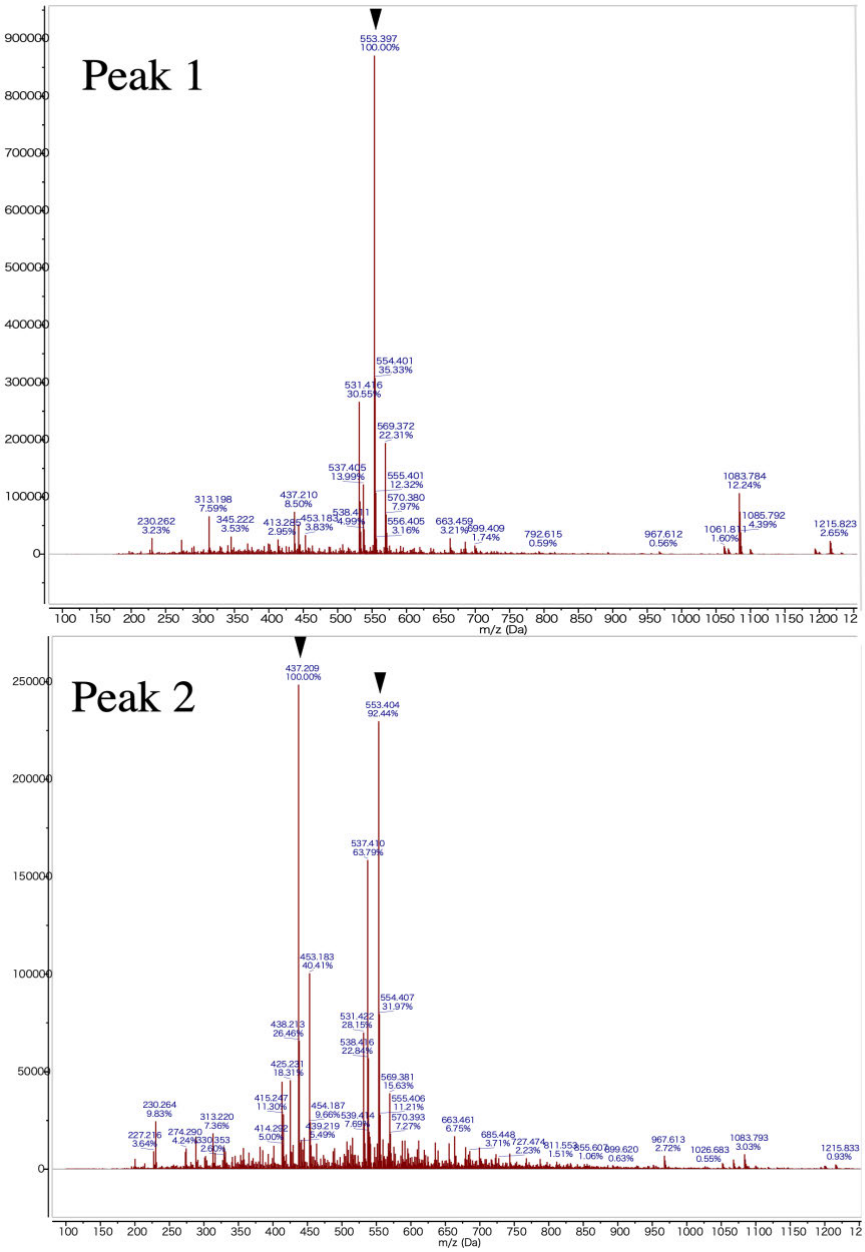


Figure 22. ESI-MS spectrum: Compounds in Peak 1 and Peak 2 were analyzed by ESI-MS. Major signals are indicated by arrow heads. From the graph, both compound contained same compound.

Absorbance of the purified compound

Both compounds are fluorogenic, according to spectrometric analysis of purified fractions (Fig. 23). Peak 1 showed a relatively weak fluorescent exit at 418 nm emitting fluorescent at 668 nm (Fig. 23A). Peak 2 compound had a relatively strong fluorescent exit at 412 nm emitting fluorescent at 672 nm (Fig. 23B). The peculiar property of emitting near-infrared light with a

large gap between the excitation and fluorescence wavelengths suggested the possibility of a new fluorescent substance.

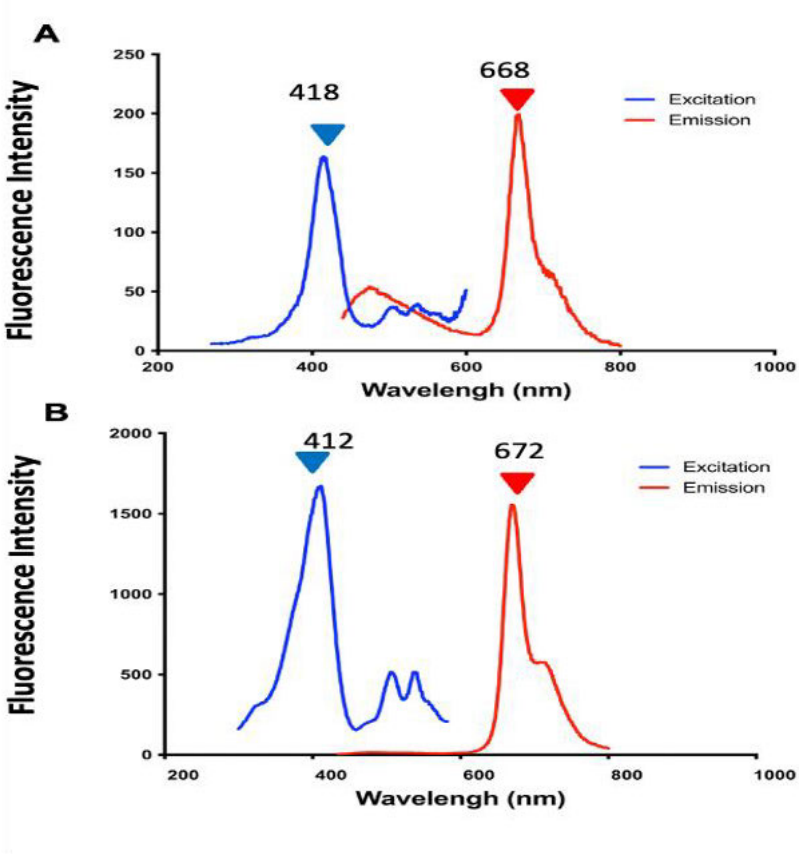


Figure 23: Absorbance scanning pattern (cyan) and fluorescent scanning pattern with excitation at maximum excitation wavelength (red) of compounds in peak1 (A) and peak2 (B). The peak of absorbance and fluorescence at are indicated by arrows.

Bio-potency of the mPR interacting chemical from *Padina* in inducing or inhibiting the fish oocyte maturation and ovulation *in-vivo*

As has already been established in previous study, several steroid-like chemical acts in inducing and preventing the fish oocyte maturation and ovulation (Tokumoto *et al.* 2005). 17,20β-DHP has been considered as a potent chemical which can intensely induce the fish oocyte maturation and ovulation. Conversely, DES another chemical which showed the strong activity to induce the GVBD but were unable to induce the ovulation (Tokumoto *et al.* 2011). Recently, Org OD 02-0 has been recognized as a new agent which can induce fish oocyte maturation and ovulation

(Rezanujjaman *et al.* 2020). In the present study, we recognized the antagonistic interface of the chemical extracted from *padina* with 17,20β-DHP. To evaluate the effects of *Padina* compound as EDC on fish oocyte maturation, we executed EtOH, DHP and extrcated chemical of *Padina* directly into the water of live zebrafish habitat. After 4 hours of incubation the morphology of zebrafish oocyte was observed under microscope. As expected, the oocyte of zebrafish became transparent after treatment with DHP, and the oocyte persisted as opaque after treatment with EtOH. The chemicals had no influence on the maturation and ovulation of fish oocytes. As a result, we found that antagonistic activity inhibited the induction of activity by the natural hormone 17,20β-DHP. Both compounds had inhibitory action on oocyte maturation produced by 17,20β-DHP in an in vitro experiment (Fig. 24 and 25A, B, C&D). Both compounds had substantial inhibitory activity on oocyte maturation and ovulation in vivo assays, with the exception of compound in peak2 which did not hinder oocyte maturation. These findings suggested that Padina-derived compounds have mPRα antagonistic action (Fig. 24 and 25 A, B, C&D).

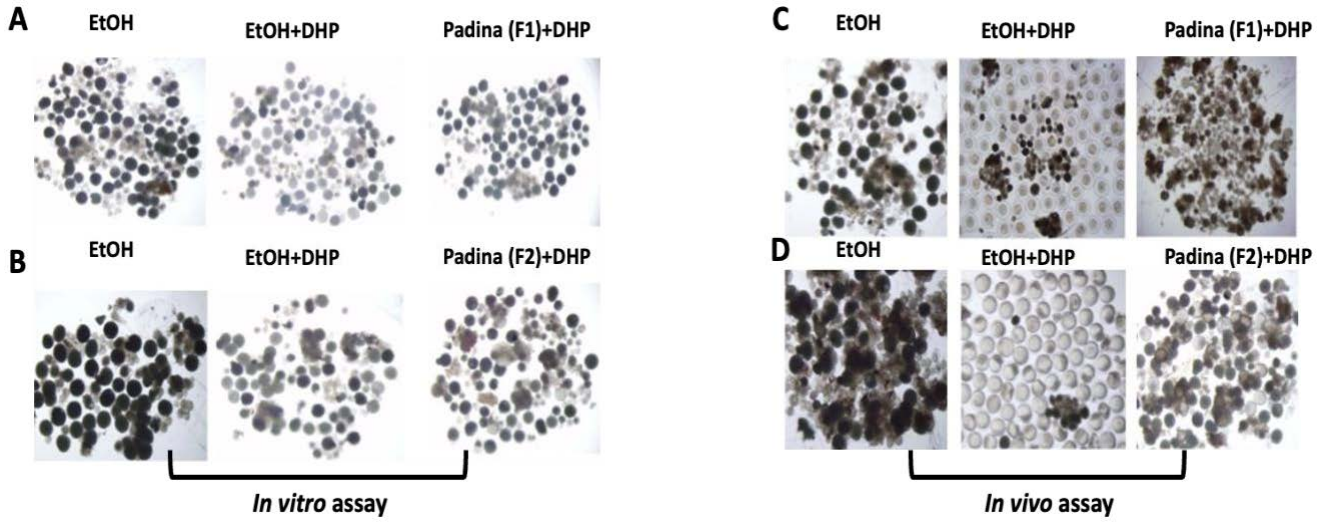


Figure 24: Morphological changes in oocytes after the action of compound from *Padina.* Panel A & B) indicated the *in vitro* assay for both compound (F1&F2). (A) showed the typical morphological changes after addition of compound (F1) with DHP, the rate of matured oocytes were decreased, (B) the compound (F2) showed the same results as agonistic to mPR. The photo- graphed were taken after the oocytes were incubated with each compound. (C & D) *In vivo* assay: the morphology of oocytes and eggs from fish treated with ethanol, 17,20β-DHP and compound from *Padina* was photographed. After 4 h of incubation, ovarian samples containing oocytes and eggs were excised from the females by dissection. The oocytes

remained opaque following treatment with ethanol. A fertilization membrane developed around eggs whose ovulation was induced by 17,20β-DHP but after the action of both compound (F1 &F2) the ovulation became stopped

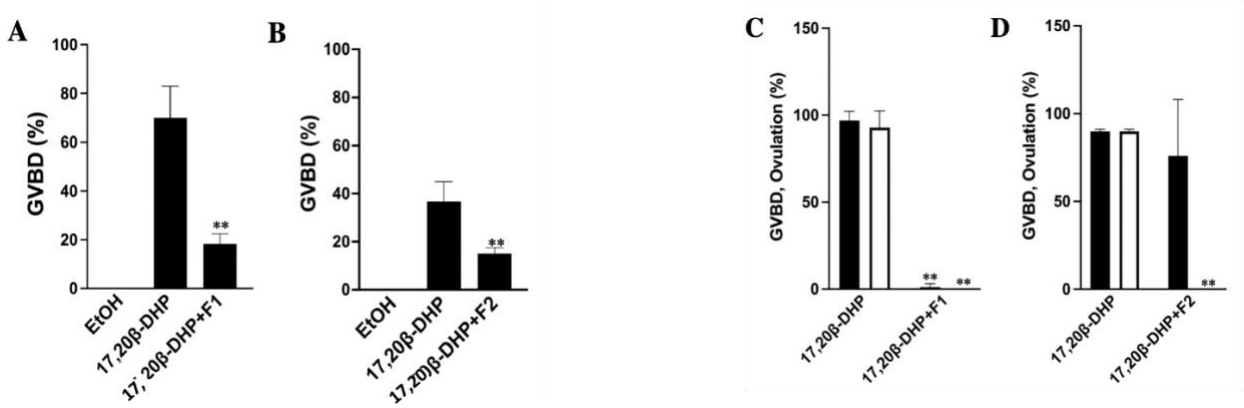


Figure 25: Purified chemicals' physiological activity on oocyte development and ovulation in zebrafish. Inhibition of 17,20β-DHP-induced oocyte maturation. The findings of in vitro assays for compounds in peak 1 (A) and peak 2 (B) are shown. With 0.1 M 17,20β-DHP, each chemical was added to the water at a final concentration of 4 μg/mL. Percent GVBD (closed column) was evaluated after two hours of incubation by scoring oocytes that became transparent. The findings of *in vivo* assays for compounds in peak 1 (C) and peak 2 (D) are shown. Percent GVBD (closed column) and percent ovulation (open column) were measured after a four-hour incubation period by scoring oocytes that became translucent and formed a fertilization membrane.

DISCUSSION

Owing to the discovery of membrane progestin receptors (mPRs) as a nongenomic signaling pathway, scientists are now exceedingly concerned to sort out the new mPR ligand as DES, DHP, and Org OD 02-0 have already been established in early research (Nagahama & Adachi, 1985, Zhu *et al.* 2003a; Zhu *et al.* 2003b, Rezanujjaman *et al.* 2020). As described in early research that maturation-inducing hormone (MIH) is a hormone that stimulates the activation of maturation-promoting factors in the cytoplasm of oocytes by acting on receptors on the oocyte membrane (Tokumoto *et al.* 2014). Several fish species have been shown to contain the two important MIHs 17,20-dihydroxy-4-pregnen-3-one (17,20β-DHP) and 17,20,21-trihydroxy-4-pregnen-3-one (20-S) (Nagahama & Adachi, 1985). As a Natural progestin, 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-DHP) has been recognized which can significantly induce oocyte maturation in fish (Nagahama & Adachi, 1985). Likewise, Diethylstilbestrol (DES), a nonsteroidal estrogen, is another endocrine-disrupting drug that induces oocyte maturation in fish through nongenomic action (Tokumoto *et al.* 2014).

A number of researchers reported that mPRs have been found in a wide range of organisms, and progestin binding activity has been discovered in numerous species, including goldfish, seatrout, zebrafish, frogs, cattle, rats, mice, and humans, from fish to humans (Thomas, 2008; Tokumoto *et al.* 2012, Josefsberg Ben-Yehoshua *et al.* 2007; Smith *et al.* 2008; Tokumoto *et al.* 2006; Tubbs & Thomas, 2009). The expression of the mPRs have been stated in reproductive tissues (ovary, uterus, and testes), kidney, brain, and spinal cord among vertebrates, including fish, mice, and humans (Hanna *et al.* 2006; Labombarda *et al.* 2010; Zhu *et al.* 2003a) which distinctly indicated the steroid-like functions of mPRs. A couple of research have already been demonstrated that the involvement of brain mPRs in mammalian sex behavior control, the contribution of mPRs in the breast tumor progression by inhibiting the cancer cell apoptosis, the roles of mPRα as a biomarker for breast cancer survival as well as its impact to generating the cancer stem cell in mammalian (Frye *et al.* 2013; Frye *et al.* 2014, Dressing *et al.* 2012, Xie *et al.* 2012, Vares *et al.* 2015).

Additionally, progesterone may elicit a rapid non-genomic response by interacting with mPRα, mPRβ, and mPRγ which stop the human T-cells from attacking the fetus (Chien *et al.* 2009). The inflammatory response and parturition are linked to progesterone signaling by mPRα,

and this link may contribute to progesterone's functional withdrawal, resulting in labor. (Lu *et al.* 2015). Now mPRs have been proven as potential cellular mediators of a variety of progesterone responses, consequently, many researchers currently exploring for new medications to treat disorders including malignancies of the reproductive tract and encephalitis.

Identification of new hormonal compounds from natural resources that can interact with mPR as novel pharmaceutical candidates or EDCs would be a massive breakthrough in reproductive or hormone research. With the rapid expansion of marine biotechnology, scientists are becoming increasingly interested in discovering marine organisms that can produce useful compounds.

However, previously we already succeeded to detect steroid-like compounds from coral reef seawater for the first time at Albion, Mauritius. The samples were fractionated by high-performance liquid chromatography to identify the chemical with mPR activity through the steroid-binding assay. The potent reactivity was observed in samples from the lagoon and reef (Tokumoto *et al.* 2017).

In the present investigation, we selected another marine alga (*Padina*) to screen out the potential chemical as a natural receptor-ligand against the mPR. As already established that the wide range of Padina can be found in the intertidal to subtidal zone, down to depths of 30–40 m, in warm temperate to tropical oceans (Win *et al.* 2021). Therefore, the samples were collected in the summer (March) from the Shimoda sea. The *Padina* samples were planted in the artificial habitat which was prepared in the marine research laboratory (a sister concern of Shizuoka University) at Mouchimoni. The extracted samples from *Padina* were separated by HPLC and some of the peaks showed compelling binding activity against mPR. To confirm the natural ligand of mPR, several purification steps were performed by using different HPLC columns as well as the mPR binding affinity of the purified chemical was approved by steroid bind assay. Electrospray ionization mass spectrometry (ESI-MS) analysis indicated the molecular mass of two purified compound, peak 1 containing the compound with the molecular mass of 554 Dalton and peak 2 containing the compound with the molecular mass of 554 and 437 Dalton. According to the ESI-MS analysis, it has been confirmed that the *Padina* species can secrete specific compound which have ability to interact with mPR cell. In additional, both compound showed fluorescence properties that clearly indicates that the compound contained fluorophore. This fluorophore compound mainly re-emit light upon light excitation. Generally,

fluorophores are the compound of the several combined aromatic groups or planar or cyclic molecules.

Meanwhile, the most noteworthy findings of the current study were to unveil the antagonistic activity of the chemical extracted form *Padina* with the most potent steroidal chemical DHP. Generally, DHP can induce the fish oocyte maturation and ovulation but surprisingly the *Padina* chemical completely hindered the ovulation process by the action of DHP when we applied *Padina* chemical and DHP combinedly into the water of fish habitat.

Identification of mPR interacting chemicals as antagonist of DHP in ovulating the fish eggs from marine algae is completely a new invention in reproductive biology, pharmacology, physiology and medical sector as well. However, previously, a group of researchers stated that macroalgae have cytotoxic action against various cancer cell models, and some authors have suggested that algae consumption could be used as a cancer chemo-preventive agent. The extract of *Padina pavonica* exhibited anti-proliferative and pro-apoptotic activities against osteosarcoma, SaOS-2 and MNNG (Kamenarska *et al.* 2002, Taskin *et al.* 2010, Bernardini *et al.* 2018).

CONCLUSION

Identification of new compound from marine organisms which may have the attributes as steroidal compound was the prime target of our study. Now a days, medical science and pharmaceuticals companies are demanding new candidates for formulating the new drugs that way scientists are focusing on the identification and characterization of such chemical from the natural resources. In our study, we curried out a pilot plant experiment on marine algae *Padina.* As our first attempt, we collected compound from *Padina* species and found very potential mPR interacting characteristics. Then we established their long-term breeding condition in the laboratory aquarium by providing the same condition as their natural habitat. Two purified compounds were found with mPR interacting ability and showed the fluorogenic characteristics as well. After determining the molecular mass of the compound by ESI-MS analysis, it can be said that the two compound share the same components. Most importantly, the purified compounds inhibited the oocyte maturation and ovulation in zebrafish which was revealed by physiological assay. However, the chemical structure of the compound not yet determined. But based on the characteristic and the bio-potency of the compound, we can say that the compounds from the *Padina* would be a new pharmaceutical candidate as an antagonist of mPRα. Determination of chemical structure of the compound would be a new challenge for us in future.

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