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In vivo and *in silico* investigation of effects of ethanol extract of *Moringa oleifera* leaves on female fertility, using fruit flies and molecular docking

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Abstract

Background: Infertility is an important issue for couples that may cause various psychological and emotional problems. Female infertility disorders play a major role in approximately 50-80% of the causes of infertility in various areas in Nigeria. *Moringa oleifera* has been proposed as a plant with female fertility enhancement effects. The objective of this study was to assess the fertility-improving effects of ethanol extract of *M. oleifera* leaf and to determine the phytochemical components causing these effects by in silico analyses.

Methods: The in vitro effects on fertility were evaluated using *Drosophila melanogaster* (fruit fly) because of its genetic similarities to humans. The copulation duration, mating latency, and the number of emergences from the fruit fly after mating were determined. Three doses (0.025%, 0.05%, and 0.1% w/w) of the *M. oleifera* ethanol extract were administered to three different groups, while a control group only received feed mixed with ethanol. For in silico studies, 62 compounds were obtained from the PubChem library by mining compounds from articles related to *M. oleifera*. Next, a ligand library was generated and docked against various targets of interest (estrogen, progesterone, kisspeptin, liver X, PPARG, and 15-PGDH receptors as well as 17β hydroxysteroid dehydrogenase and insulin-degrading enzymes) which have female fertility-enhancing effects.

Results: The in vivo experiments showed that *M. oleifera* had no effect on copulation duration and mating latency, but interestingly, it enhanced the fertility/emergence of the treated fruit flies. In silico studies suggested that phytochemicals such as rutin, marumoside B, myricetin, and quercetin showed docking scores that may well support previous works on *M. oleifera* enhancement of female fertility.

Conclusion: The results showed that *M. oleifera* can enhance fertility in female fruit flies.

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Drosophila melanogaster
3 (or 17)-beta-hydroxysteroid dehydrogenase
In silico model

Article Type: Original Article

Highlights

What is current knowledge?

The high level of fertility in treated flies was dose-dependent.

What is new here?

Rutin and myricetin present M. oleifera are responsible for the female fertility enhancement effect.

Introduction

Reproduction is one of the most important traits of all living organisms, which is crucial for the continuation of life. Infertility has continued to be an issue of concern for couples and sexually active people who want to have children, and their search for a therapeutic solution has proven to be sometimes fruitless. A condition of the reproductive system known as infertility is characterized by the inability to achieve a clinical pregnancy after at least a year of regular, unprotected sex without complications like lactation or postpartum amenorrhea. Infertility affects about 10% of people of reproductive age (1) and 15% of couples globally, 50% of which are female-related alone (2). This condition can be related to both innate factors (genetic, immunological, anatomical, and hormonal disorders) and external factors (obesity, infections after childbirth or surgery, tuberculosis of the pelvis, and obesity) (3). For couples, infertility is a major cause of concern, and in various areas of Nigeria, female infertility accounts for 50-80% of all divorces (4). Female infertility has been linked to several medical conditions including pelvic inflammatory disease, polycystic ovarian syndrome, endometriosis, premature ovarian failure, uterine fibroids, and environmental toxins such as glues, volatile organic solvents, silicones, organic dust, pesticides, and physical agents (5). Ovulation and uterine problems, tubal blockages, hormonal imbalances, prior tubal ligations, and weight changes that can lead to ovarian dysfunction are possible additional causes of infertility in females (6). In addition, a low body fat percentage impairs the production of estrogen, which results in an aberrant ovulation cycle and irregular menstruation (7).

Moringa oleifera is a plant from the family Moringaceae with a wide range of medicinal applications (8). It has been also used in the production of pharmaceutical and industrial products and therefore has been regarded as the "miracle tree" (9, 10). The phytochemical screening of M. oleifera leaf extract has demonstrated the presence of alkaloids, saponins, tannins, steroids, phenolic acids, glucosinolates, flavonoids, and terpenes (11). Numerous researches in Nigeria have shown that M. oleifera contains both macro- and micronutrients that can be used for the treatment of female infertility (12-14). The plant has been thought to improve female fertility by increasing the secretion or availability of ovarian hormones, such as progesterone and estrogen, which are in charge of pregnancy maintenance, ovulation, implantation, and delivery (15, 16). This study aimed to assess the impact of ethanol extract of M. oleifera leaf on the reproductive potential of fruit flies. Several organisms share the fruit fly's basic genetic mechanism, and hundreds of closely similar species have been thoroughly investigated for decades, with a wealth of literature available (17). In this study, molecular docking analysis was used to identify M. oleifera's molecular target activity.

Methods

The materials used include breeding and treatment vials, a micropipette, empendox tubes, cotton wool, a stirring rod, a spatula, a pot, ethanol, sample bottles, a beaker, a measuring cylinder, distilled water, filter paper, funnel, an electric cooker, water bath, and blender.

Preparation of M. oleifera leaf

Fresh *M. oleifera* leaves were purchased from a nearby plantation in Mararaba, Nasarawa State, Nigeria. Botanists identified the plant leaves, assigned them to herbarium number 08, and let them air dry at room temperature. The dried leaves were powdered and soaked in 250 ml of ethanol for 72 hours. After filtration, the mixture was dried in a water bath (B-Bran Scientific and Instrument Company, England) at 60ºC. As described previously (18), the brownish residue was cleaned and refrigerated until used in an airtight bottle.

Feed preparation and dose concentrations

Feed preparation was carried out as explained previously (19). The feed was then well stirred into a mixture, weighed into the treatment vials, covered, and left to cool naturally.

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Preparation of offspring used for the study

Flies were bred on corn agar media. The eggs hatched into adult fruit flies after 7 days of breeding. After approximately 10 days of breeding, the stock generation of flies was removed, and the daughter generation (offspring) was allowed to acclimatize at 25 ± 2°C until the needed number of flies for treatment was obtained. Four groups of 25 flies (both sexes) were used for the investigation. Each group received three duplicates and received the ethanolic extract of *M. oleifera* at three distinct concentrations: 0.025%, 0.05%, and 0.1% w/w. Dead flies were counted and recorded every day, and the percentage of surviving flies was calculated up until the point at which all insects were dead.

Survival test and lethal concentration 50 (LC50) determination

Four separate groups (A, B, C, and D), each containing 25 flies, received 0, 0.05%, 0.1%, and 0.2% w/w of the extract incorporated in the feed for 28 days. The groups were examined daily for dead flies. Next, the subacute median lethal concentration (LC_{50}) was determined as described in a previous study (19).

Fruit fly preparation

The fruit flies for the fertility and survival studies of *M. oleifera* consisting of both males and females were obtained from the Pharmacology and Toxicology Department of Madonna University, Elele, Rivers State. The fruit flies were bred. The study was carried out after obtaining the approval of the Animal Research Ethics Committee, Madonna University, Elele, Nigeria (EC/FP/023/14).

Mating experiment

Less than 8 hours after enclosure, 25 male flies were randomly divided into 4 distinct vials (n=6) marked A (untreated), B, C, and D. Various concentrations of the ethanol root extract of *M. oleifera* (0.025%, 0.05%, and 0.1% w/w) were added to the feed for groups B-D. One virgin female and one treated virgin male were coupled in one vial for each group. After the flies were coupled, the mating activity was observed for one hour. For each of the 24 pairs, the length of the copulation was scored.

Fertility studies

The female flies were allowed to lay eggs for 2 days after each pair was moved to a fresh agar-corn meal diet. Then, the pair was moved into another treatment vial with fresh feed for an additional 2 days. The parent flies were then disposed of by placing them in a methanol-filled bottle. After 8 days, the offspring from each replicate vial labeled A1–A6, B1–B6, C1–C6, and D1–D6 were scored for 7 days until no more emergence occurred (19).

Ligand library generation

To create the ligand library, identified secondary metabolites of *M. oleifera* were selected from published literature. In Standard Database Format (2D), 60 secondary metabolites were obtained from the NCBI PubChem database. The resulting ligand library was produced using the docking software Maestro (Schrodinger suite, version 2018-1b) as described in a previous study (20).

Protein preparation

With the help of their respective protein databank (PDB) IDs, a variety of targets were obtained from the PDB along with their associated co-ligands. These included the estrogen receptor co-ligand (1FDT), progesterone receptor co-ligand (1A28), liver X receptor co-ligand (3LOE), peroxisome proliferator-activated receptor gamma (PPARG) co-ligand (6KOT), the standard agent for 15-hydroxyprostaglandin dehydrogenase (15-PGDH)_SW 033291 (2GDZ), 17 beta-hydroxysteroid dehydrogenase co-ligand (5L7Y), and insulin-degrading enzyme co-ligand amylin (3HGZ) as described previously (21) using the Protein Preparation Wizard (22).

Statistical analysis

GraphPad Prism (version 5.01) was used for the statistical analysis.

Results

Survival studies of M. oleifera on D. melanogaster

The results revealed that the survival rates of the *M. oleifera* ethanol leaf extract treatment groups (B, C, and D) did not differ significantly compared to that of the control group A (Figure 1).

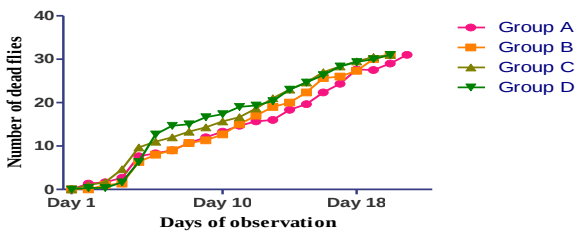

Figure 1. Survival studies of *M. oleifera* on *D. melanogaster*

Effect of M. oleifera on mating latency

Mating latencies of the treated groups differed significantly compared with the control group. However, the mating latencies of the treated group were longer at the lowest dose (50 mg/kg, group B) (Figure 2).

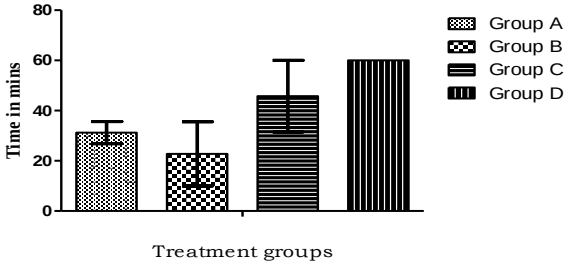

Figure 2. Effect of M. oleifera on mating latency

Copulation duration

There was a great decline in the copulation durations in a dose-dependent manner. No mating or copulation was observed within an hour of observation in group D (the highest-dose treatment group) (Figure 3).

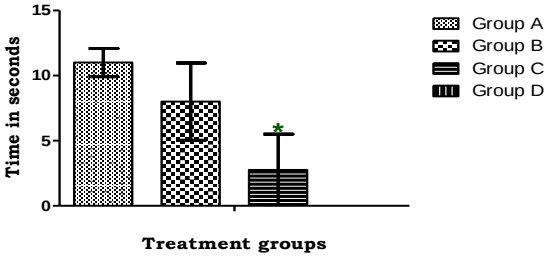

Figure 3. Effect of *M. oleifera on* copulation duration (*P<0.05 *M. oleifera* versus the control group)

Effect of M. oleifera on the emergence of flies

The result showed a paradoxical but interesting effect. We detected a high fertility level and fecundity level in the treated flies. Nevertheless, there was no statistically significant difference between the groups in terms of fertility level (Figure 4).

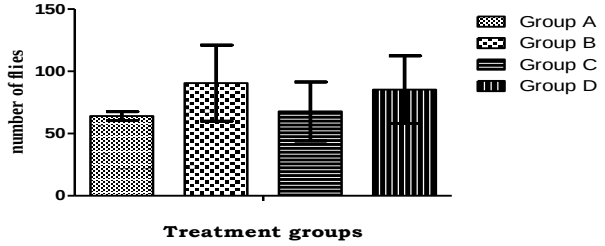

Figure 4. Effect of M. oleifera on the emergence of flies

Molecular docking study

Figures 5 to 12 show the results of molecular docking study on estrogen receptor, progesterone receptor, kisspeptin receptor, PPARG, 15-PGDH, 17β hydroxysteroid dehydrogenase, insulin-degrading enzyme, and liver X receptor.

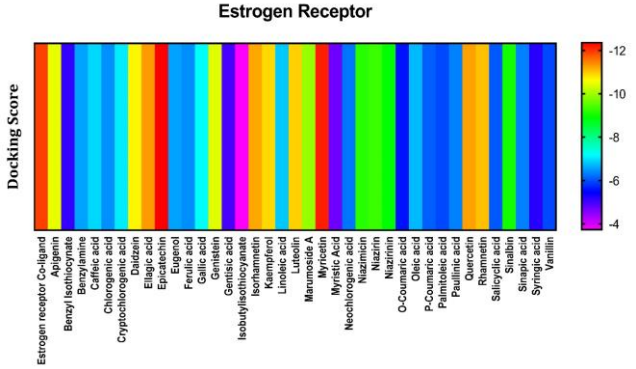

Figure 5. Heat map representation of docking result for compound interaction with the estrogen receptor (-4 kcal/mol) to red (-12 kcal/mol)

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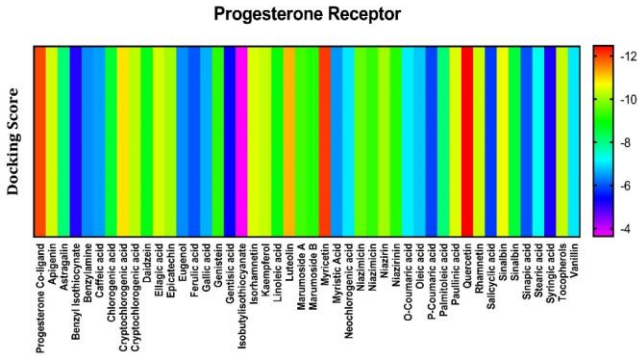

Figure 6. Heat map representation of docking result for compound interaction with the progesterone receptor (-4kcal/mol) to red (-12kcal/mol)

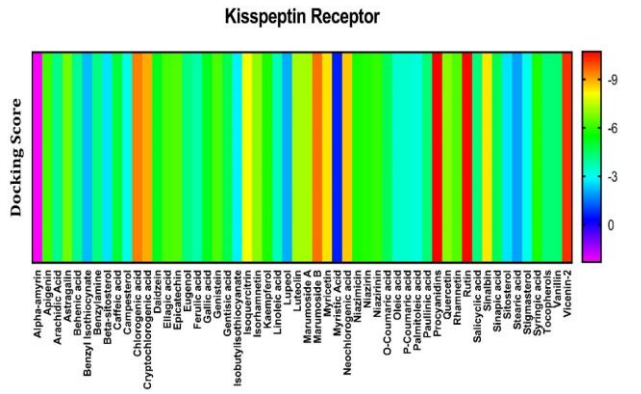

Figure 7. Heat map representation of docking result for compound interaction with the kisspeptin receptor (-0kcal/mol) to red (-9kcal/mol)

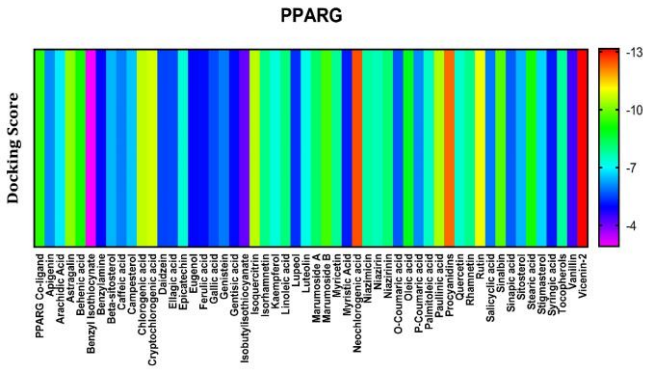

Figure 8. Heat map presentation of docking result for compound interaction with PPARG (-4kcal/mol) to red (-13kcal/mol)

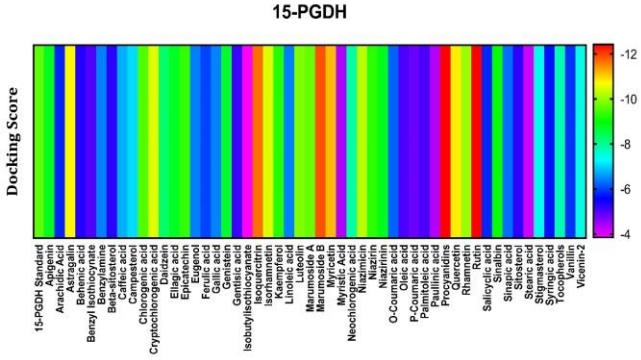

Figure 9. Heat map presentation of docking resulting from compound interaction with 15-PGDH (-4kcal/mol) to red (-12kcal/mol)

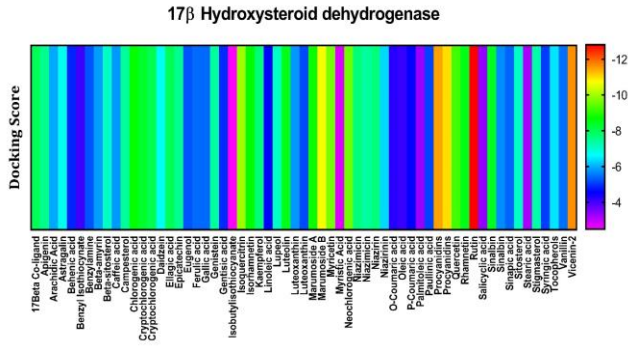

Figure 10. Heat map presentation of docking resulting from compound interaction with 17β hydroxysteroid dehydrogenase (-4kcal/mol) to red (-12kcal/mol)

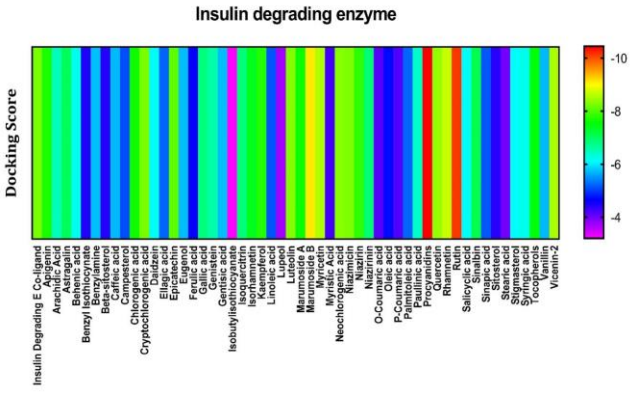

Figure 11. Heat map presentation of docking resulting from compound interaction with the insulin-degrading enzyme (-4kcal/mol) to red (-10kcal/mol)

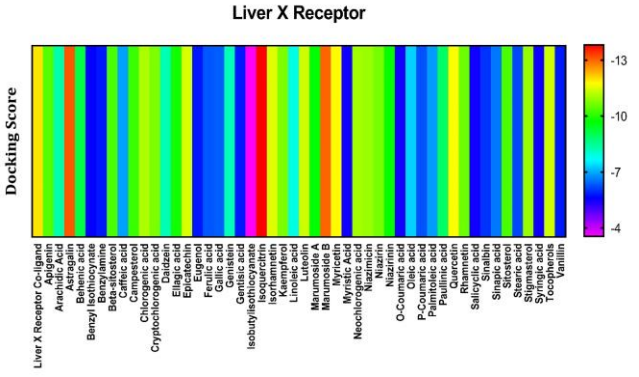

Figure 12. Heat map presentation of docking resulting from compound interaction with Liver X receptor (-4kcal/mol) to red (-13kcal/mol)

Discussion

For couples and sexually active people who desire to have children, infertility has continued to be a major hurdle and health issue. In underdeveloped nations where manufactured treatments are expensive and difficult to obtain, the use of medicinal plants for infertility treatment might be a suitable alternative (23). Orthodox drugs such as clomiphene, tamoxifen, gonadotropins, metformin, letrozole, and bromocriptine have some undesirable effects. These side effects have prompted research on the use of medicinal plants to solve female infertility problems. Numerous investigations on the effect of *M. oleifera* on female fertility have revealed that the plant contains phytoestrogens, which exert estrogen-like effects. Treatment with *M. oleifera* ethanol extract on rats for 30 days resulted in a significant rise in luteinizing hormone and estrogen contrary to the follicle-stimulating hormone level, which remains unchanged (15). The extract's activation of estrogen receptors is assumed to be the cause of the elevated levels of estrogen and luteinizing hormone. The compounds present in the extract can bind to estrogen receptors to bring about estrogenic responses (15).

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According to the literature, the effects of ethanol extract of *M. oleifera* on the ovary may be secondary to their basic actions on the hypothalamus or pituitary gland. This results in the amplification of normal gonadotropin release (16, 24). The administration of *Moringa* leaves at 200 and 500 mg/kg body weight for 30 days significantly increased the number of implantation sites on the 8th day of pregnancy in rats. No implantation site was observed in the high- and low-dose group of rats administered with other parts of the *Moringa*, namely the seed, flower, root, and seed. The findings suggested that the consumption of *M. oleifera* leaf extract increases female reproductive function and fecundity in rats, as noticed by the increase in the number of embryos successfully implanted in the rats' uterus. According to the results of this study, *Moringa* leaves may improve female reproductive function by boosting the availability or release of ovarian hormones such as progesterone and estrogen. This suggests that *M. oleifera* ingestion is safe and does not result in fetal abortion.

In the present study, fruit fly was used for in vivo experimentations because of their genetic similarities to humans, very short life cycle, and production of a large number of offspring, which allow sufficient data collection. We determined the survival of the fruit fly using different concentrations of the extract and measured copulation duration, mating latency, and emergence of the flies after mating. The lack of significant difference in the survival rate of subjects between the treated groups and the control group might indicate that *M. oleifera* does not affect the survival rate of fruit flies. The mating latencies of the treated groups were longer; therefore, its lowest dose may be safer. This may be an indication that this agent is not likely to have aphrodisiac properties due to a notable decline in the copulation durations in a dose-dependent manner. The emergence of fruit flies could indicate the fertility enhancement effect, showing that there was a high level of fertility and fecundity in treated flies in a dose-dependent pattern. These positive results provided the need to ascertain the different targets to predict the pro-fertility potentials of *M. olifeira* component by conducting in silico studies. As mentioned previously, *M. oleifera* has been adjudged to contain macro and micronutrients that are potentially beneficial for the treatment of female infertility (12-14). Phytochemical screening of *M. oleifera* has previously revealed the presence of saponins, alkaloids, steroids, tannins, steroids, phenolic acids, glucosinates, terpenes, and flavonoids (11). In this study, virtual screening of *M. oleifera* constituents from the literature was carried out, and the following compounds were obtained: rutin, apigenin, isorhamnetin, quercetin, luteolin, genistein, daidzein, myricetin, epicatechin, sinalbin, salicyclic acid, gentistic acid, syringic acid, caffeic acid, sinapic acid, o-coumaric acid, p-coumaric acid (25), isoquercetin, astragalin, crytochlorogenic acid (26), procyanidins (27), beta-amyrin, alpha-amyrin, neochlorogenic, rhamnetin (28), vicenin-2 (29), benzyl isothiocyanate, oleic acid, myristic acid, palmitic acid, isothiocyanate (30), E-zeaxanthin, Z-lutein, E-lutein, E-luteoxanthin, beta-carotene (31), gallic acid, ellagic acid, ferulic acid, chlorogenic acid (32), paullinic acid, behenic acid, linoleic acid, beta-sitosterol, arachidic acid, stearic acid, palmitoleic acid, stigmasterol, campesterol (33), lupeol, vanillin (34), eugenol (35), niazirin, niaziridin (36), rhamnopyranoside (37), niazimicin (38), and niaziminin (39). Some phytochemical compounds present in *M. oleifera,* such as rutin, quercetin, isorhamnetin, lupeol, vicenin-2, chlorogenic acid, vanillin, isoquercetin, and cryptochlorogenic acid have been shown to possess fertility effects by enhancing ovarian folliculogenesis to bring about ovulation, enhance steroidogenesis by binding to their nuclear receptors, regulating some enzymes of steroidogenesis or regulating the activities of key enzymes involved in their metabolism (40). Such regulator effect on the reproductive function can be directly linked to its influence on the hypothalamic-pituitary-ovarian axis by stimulating or inhibiting the enzymes and receptors involved in this process and steroidogenesis disrupting the hormonal functioning of the hypothalamus and pituitary gland. Some receptors and enzymes must be stimulated or inhibited to improve female fertility since they are responsible for follicle development, oocyte maturation, fertilization rates, pregnancy outcome, embryo development, and early implantation. The receptors with this fertility effect include progesterone receptor, estrogen receptor, kisspeptin receptor, liver X receptor, and peroxisome proliferator activators receptor, and the enzymes include 15-hydroxy prostaglandin dehydrogenase, 15-beta hydroxysteroid dehydrogenase, and insulin-degrading enzyme.

The fertility potential of rutin, cryptochlorogenic acid, luteolin, myricetin, Isorhamnetin, quercetin, marumoside B, vicenin-2, isoquercetin, sinalbin, and astragalin present in *M. oleifera* was established by the stimulatory effect on the progesterone, estrogen, Kisspeptin, liver X, and PPARG receptors and the inhibitory effects on 15-hydroxy prostaglandin dehydrogenase, 15-beta hydroxysteroid dehydrogenase, and insulin-degrading enzymes, all of which are involved in female fertility. Molecular docking studies are based on the fact that compounds possess the capacity to bind to amino acid residues, which is a function of protein conformation and the assumed pose of the ligand (41). The library of phytochemicals derived was docked against the five target proteins, which may be remedial in infertility in females.

Estrogen and progesterone receptors are essential since both are the functional key hormones for female fertility. In other words, stimulation of these hormones enhances fertility in females. In the docking studies, heat map representation revealed that myricetin has a high stimulatory docking affinity for estrogen receptors significantly higher than that for the co-crystallized ligand at −12.155kcal/mol. With a docking score of -12.362 kcal/mol, epicatechin showed a similar potential to myricetin to that of the co-ligand, which was -11.933 kcal/mol. Quercetin may also stimulate progesterone receptors as it possesses a high docking score of -12.477 kcal/mol, and myricetin at -12.086kcal/mol is more than the co-ligand at -11.988 kcal/mol. When stimulated, the kisspeptin receptor also causes the secretion of gonadotropins, including luteinizing hormone (LH) and follicle-stimulating hormone, which enhances female fertility (42). The docking studies revealed that rutin stimulates the kisspeptin receptor more than the co-ligand at 11 kcal/mol.

Peroxisome proliferator-activated receptors also play a role in female fertility and reproduction, which increases the pituitary production of prolactin and LH (43), thereby assisting folliculogenesis. The heat map representation revealed that vicenin-2 may stimulate PPARG significantly and even more than the co-crystallized ligand at −13.155kcal/mol, while rutin may stimulate PPARG at −11.008 kcal/mol compared to the co-ligand at -9.39 kcal/mol.

Stimulation of prostaglandin E2 receptor plays a role in female fertility by increasing LH levels, thereby enhancing ovulation, implantation, and menstruation (44). Enzymes that degrade prostaglandins such as 17β hydroxysteroid dehydrogenase and 15-PGDH also play a role in enhancing female fertility. Inhibiting this enzyme that metabolizes prostaglandin leads to an increased level of prostaglandin and thus can facilitate the actions of prostaglandin in fertility enhancement in females. Heat map representation also revealed that rutin may inhibit the 15-PGDH enzyme significantly even more than the co-crystallized ligand at −12.428 kcal/mol, and procyanidins had a close effect on rutin with inhibition of 15-PGDH at −12.428 kcal/mol compared to the co-ligand at -9.635 kcal/mol. Rutin also may inhibit 17β hydroxysteroid dehydrogenase at -12.813 kcal/mol and procyanidins at -11.497 kcal/mol more than the co-ligand at -7.989 kcal/mol.

Insulin-like peptide 3 (INSL3) receptor plays a role in increasing progesterone production (45) and oocyte maturation (46). Insulin-degrading enzymes must be inhibited to increase the level of insulin for this effect. The results obtained revealed that rutin may inhibit insulin-degrading enzymes significantly even more than the co-crystallized ligand at −10.134 kcal/mol. Marumoside B may also inhibit insulin-degrading enzymes at −9.111 kcal/mol compared to the co-ligand at -8.236 kcal/mol.

Stimulation of the liver X receptor influences cholesterol metabolism, which is the precursor for ovarian steroid biosynthesis (47). The results showed that isoquercetin stimulates the liver X receptor at -13.813 kcal/mol, marumoside B at -13.016 kcal/mol, and astragalin at 3.129 kcal/mol even more than the co-ligand at -11.962 kcal/mol.

Our results obtained from the molecular docking studies indicate that rutin and myricetin found in *M. oleifera* have the potential to produce the best effect in almost all receptors and enzymes involved in the fertility process.

Conclusion

The ethanol extract of *M. oleifera* leaf shows less effect in copulation and mating latency but affects the number of fruit flies' emergence after mating, supporting the claim that *M. oleifera* has a female fertility-enhancing effect**.** Effective stimulation of the estrogen, progesterone, and PPARG receptors and inhibition of 15-PDGH, insulin-degrading, and 17β hydroxysteroid dehydrogenase enzymes indicate the potential of this extract for female fertility enhancement, which can be mainly attributed to rutin and myricetin.

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Ethical statement

The study was carried out after obtaining the approval of the Animal Research Ethics Committee, Madonna University, Elele, Nigeria (EC/FP/023/14).

Conflicts of interest

There is no conflict of interest regarding the publication of this article.

Author contributions

E B.E designed and statistically analyzed the data for the study. The initial draft

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of the manuscript was written by S. K. M. Laboratory tests and literature searches for methodology were managed by O.U.I. and U.L.I. The final manuscript was reviewed and approved by all writers.

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