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In Silico Studies for Multi-Targeting *Justicia Secund* Phytochemicals Against Ovarian Cancer

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ABSTRACT

Introduction/Background of study: Ovarian cancer is the fifth leading cause of cancer deaths among women, accounting for more deaths than any other female gynaecological cancer, which includes cervical, uterine, vaginal and vulvar cancers. Current treatment options include surgery, platinum-based chemotherapy, radiation therapy and the use of targeted therapy such as poly ADP-ribose polymerase (PARP inhibitors), as well as immunotherapy. These therapy options, however, are subject to high rates of resistance and many side effects.

Aim/ Objectives: This research aims to study other viable drug targets for ovarian cancer treatment, as well as new phytocompounds that can serve as new drug options using the in-silico approach.

Materials/Methods: The ethanolic leaf extract of *Justicia secunda* was obtained using conventional methods. Liquid Liquid-liquid fractionation was performed with N-hexane, ethyl acetate and butanol solvents to obtain their fractions alongside the aqueous fraction. Vacuum Liquid Chromatography was performed with the N-hexane fraction and gradient mixtures of N-hexane/ethyl acetate and Dichloromethane/Methanol in various ratios to obtain the subfractions. The compounds were identified using Gas Chromatography-Mass Spectrometry (GC-MS). Full pharmacognostic profiling was performed on the *J.secunda* leaves. The identified compounds were downloaded from Pubchem and subjected to Molecular docking simulations to obtain their binding affinities with the receptors of interest. Drug-likeness and toxicity assessments were performed on frontrunner compounds.

Results: After assessment of the frontrunner compounds, four multitargeting *J.secunda* phytochemicals were identified: Luteolin, Diosmetin, 5H-Quindoline and 10H-Quindoline.

Conclusion: This study shows that some phytocompounds in *J.secunda* have better binding affinities and possibly better interaction against RTKs overexpressed in ovarian cancer, when compared to the reference drugs currently in use. Further in-vitro and in-vivo studies are recommended to ascertain if these compounds have any inhibitory activity against these receptor tyrosine kinases.

KEYWORDS: *Justicia secunda*, Ovarian cancer, RTKs, ligands, receptors, phytocompounds.

1. INTRODUCTION

Ovarian cancer is a gynaecological malignancy that occurs as a result of uncontrolled growth of abnormal cells in the ovaries, the organs that produce eggs in the female, or in the related areas of the fallopian tubes and peritoneum. The exact aetiology of most ovarian cancers is still not known to cancer researchers. At the outset of ovarian cancer, just like in many cancers, there are usually few or no noticeable symptoms, but if these symptoms occur, they can be like those seen in other conditions such as premenstrual syndrome, irritable bowel syndrome, or a temporary bladder problem. However, if these symptoms are due to ovarian cancer, the symptoms will persist and worsen(Radu et al., 2023) . Ovarian cancer as a disease has poor prognosis and unsatisfactory therapeutic outcomes because the current treatments are susceptible to failures, leaving the patients with no other options for treatment. The late detection of the disease and resistance to pharmacotherapy, mostly platinum compounds, are the main reasons for poor prognosis. About a third of patients do not respond to primary platinum-based chemotherapy treatment, and over time, eventually, 80% of other patients develop resistance to chemotherapy, making room for disease reoccurence (Lukanović et al., 2022). Therefore, it is imperative to keep working on new and better treatments for this malignancy. In this study, we aim to discover multi-targeting *Justicia secunda* phytochemicals that inhibit the overexpression of four (4) known receptor tyrosine kinases expressed in ovarian cancer, namely; Epidermal growth factor receptors (EGFRs), Vascular endothelial growth factor receptors (VEGFRs), Hepatocyte growth factor receptor (HGFR), and Fibroblast growth factor receptors (FGFRs) using in-silico studies. Past and recent studies have shown that inhibition of overexpressed RTKs in ovarian cancers is a promising line of treatment. Researchers have developed many inhibitors that are currently in use, while some others are currently undergoing clinical trials.

Here we briefly review the four RTKs of interest, their roles in the progression of cancer and how their inhibition can be beneficial to ovarian cancer treatment.

1.1 EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR)

The epidermal growth factor is a family of receptor tyrosine kinases (ErbBs) that plays essential roles in the regulation of cell growth or proliferation, survival, differentiation and migration. They also play a critical role in the multiplication of cancer cells. It is a transmembrane protein that belongs to the ErbB family of receptors that are overexpressed in various cancers. EGFR is composed of four members that are similar in structure and cellular functions: ErbB1 (EGFR or HER1), ErbB2 (HER2), ErbB3 (HER3) and ErbB4 (HER4) (Balogun et al., 2021). Dysregulation in EGFR activation leads to an assembly of signaling complexes and stimulation of many downstream signaling cascades associated with cell growth and survival, increased angiogenesis, and metastasis in tumors (Ngaha et al., 2023). A review by Sheng and Lui however showed that the number of Ovarian cancers with EGFR activating mutations and amplification are small (<4% and 4–22%, respectively) compared to other cancers such as lung cancers (Rendell et al., 2022).

1.2 VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR (VEGFR)

Vascular endothelial growth factor receptors (VEGFRs) are a family of receptor protein tyrosine kinases that play an important role in the regulation of tumor-induced angiogenesis (Liu et al., 2022). Angiogenesis is the growth of new blood vessels from the existing blood vessels. The ability of tumors to form new blood vessels has been a major focus of cancer research over the past few decades. VEGF also plays an important role in tumor metastasis by causing the construction of abnormal blood vessels. High degrees of tumor angiogenesis and VEGF expression in ovarian cancer are associated with poor disease outcomes. Due to the vital role of VEGF in promoting various cancers, its signaling pathway has been studied as an attractive target for cancer therapy. It has been shown that VEGF blockage normalizes tumor vessels and increases oxygen and chemotherapeutic agents' delivery into tumor tissue (Ranjbar et al., 2015).

1.3 FIBROBLAST GROWTH FACTOR RECEPTOR (FGFR)

Fibroblast growth factor receptors (FGFRs) are a family of receptor tyrosine kinase (RTKs) encoded by four different genes (FGFR1-4). The activation of FGFR is involved in cell proliferation, differentiation, tissue modeling, and angiogenesis through gene amplification, overexpression, point mutations or chromosomal translocations, which can lead to the development and/or progression of cancer (Zhu et al., 2020). In the last few years, many studies have shown increasing evidence that FGFRs are important oncogenes in certain cancers and act in an independent way to maintain the malignant properties of tumor cells. These observations make FGFRs very attractive as targets for therapeutic intervention in cancer. Since FGFR inhibition can reduce proliferation and induce apoptosis in a variety of tumor models showing FGFR aberrations, many researchers have selected FGFRs as targets for anticancer drug development.

1.4 *JUSTICIA SECUNDA* VAHL

Justicia secunda Vahl. is also known as St. John's bush, bloodroot, or water willow. In Barbados, it is known as "Bloodroot" and in Venezuela as "Sanguinaria", and**a** this is due to the red color of water observed when the plant is boiled. Amongst Barbadian locals, they describe the oral use of decoctions and infusions from the leaves of this plant to treat wound infections. It is also used for bathing dogs suffering from skin rashes. A survey carried out in Kikwit city (Kwilu province, southwest part of the Democratic Republic of the Congo) showed that *J. secunda* leaves were used locally to treat sickle cell disease. In Nigeria, Congo, and southern Cote d'Ivoire, some people consume a decoction of the leaves to treat anemia. *Justicia secunda* is a medicinal plant that has been locally used as a blood tonic in Nigeria for a long time. The local users of the leaves are mainly patients with sickle cell anemia, women who want to replenish their blood after the menstrual cycle, and pregnant women. *Justicia secunda* has been proven by researchers to have more effective blood-boosting properties than blood tonics in animal models. Due to this, blood levels can be effectively restored to normal within a short time. In the Igbo language of the Southeastern Nigerians, it is known as "Ogwu Obara" (blood medicine). The Yoruba call it "ewe eje" (blood leaf). Some Justicia species are used to treat inflammation, gastrointestinal disorders, respiratory infections, fever, pain, diabetes, diarrhea, liver disease, rheumatism, and arthritis. It also has anti-inflammatory, antihypertensive, anti-bacterial, antitumor, antiviral and analgesic effects [11].

2. MATERIALS AND METHODS

Study Area: This study was carried out in the Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Agulu Anambra State, Nigeria.

Plant collection and extraction: The previously identified plant was harvested carefully from the school medicinal plants garden before sunrise. It was air dried under a fan in a room until the plant sample was completely dried. The dried plant was milled into powder using a manual miller. A 500g of the powder was added to each of the two glass jarsair-dried. The plant material was soaked in 2500 ml of absolute ethanol (95%) in each jar. The mixture was left to stand for 24 hours with agitation at intervals. The mixtures were decanted and filtered with a Whatman No. 1 filter paper. The filtrate was concentrated at a temperature of 60^{o} C using a rotary evaporator. The concentrated extract was collected in a sample bottle and stored at room temperature on the laboratory shelves.

Liquid-Liquid Fractionation: The concentrated extract was dissolved in 50 ml of ethanol and 150 ml of water. This mixture was poured into a separating funnel and subjected to liquid-liquid fractionation using partitioning technique with three different solvents: N-hexane, ethyl acetate, and butanol. After the final

separation, the resulting fractions, namely the n-hexane, ethyl acetate, butanol fractions, and the aqueous phase were concentrated using a rotary evaporator, with the temperature of the evaporator set at 60 °C.

Vacuum-Liquid Chromatography: After the liquid-liquid fractionation, the ethyl acetate fraction was subjected to a vacuum liquid chromatography (VLC) by performing a stepwise separation under vacuum using solvents with increasing polarity, starting from non-polar to highly polar solvent systems. The elution process started with using a gradient mixture of 500 ml of n-hexane and ethyl acetate prepared in various ratios (10:0, 9:1, 8:2,7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, and 0:10). After this, a gradient mixture of 500 ml of dichloromethane and methanol in different ratios (10:0, 1:9, 3:7, 5:5, 7:3, and 9:1) was used for further separation. After the separation process, the solvents in the different fractions were evaporated at room temperature using air from a ceiling fan. This procedure allowed for the isolation and collection of specific components based on their different polarities, leading to the successful fractionation of the crude material for further analysis using GC-MS.

Molecular Docking:

For the in-silico part of the study, some biological databases and journal publications were mined to find out the receptor tyrosine kinases (RTKs) that are important for the oncogenesis and progression of ovarian cancers. This was done to discover possible targets/receptors for the inhibitory phytochemicals to act on.

Selection and preparation of targets

After the literature mining, 4 targets were selected for the docking process. These targets include Epidermal growth Factor Receptor (EGFR), Vascular Endothelial Growth Factor Receptor (VEGFR), Hepatocyte Growth Factor Receptor (HGFR) and Fibroblast Growth Factor Receptor (FGFR). The 3D structures of these targets/proteins were obtained from Protein Data Bank (http://rscb.org) with the protein codes; 8A27, 6XVJ, 7V3R and 5O49 respectively. The PyMOL software was used for the initial preparation of the PDB files to select the needed chains and to delete multiple ligands and non-protein parts such as water molecules. Another software, Auto dock tools, was used to add polar hydrogens to the protein/targets, then they were exported as a PQBQT file. PDBQT is the file format recognized by the molecular docking simulator for both ligands and receptors/targets. The Auto dock tools software was used to create grid boxes of different sizes and centers around the active site of the protein.

Selection and Preparation of Ligands

A total number of 29 phytochemicals isolated from *Justicia secunda* ethyl acetate fraction were identified using GCMS. Some other previously isolated compounds from *Justicia secunda* were sourced from existing literature. These sixty-eight compounds were then obtained from PubChem (http://pubchem.ncbi.nlm.nih.gov/) in the SDF-3D format. The ligands co-crystallized with the receptor site on the proteins were used as the reference ligand/standard. Some known inhibitors of the different RTKs were also downloaded from Pubchem in the same format. Using Auto dock tools, the ligands were prepared for molecular docking simulation. All rotatable bonds and torsions were added and the ligands were saved as PDBQT files. The prepared ligands and the protein targets/receptors were used in molecular docking simulation.

Molecular Docking of the Phytochemicals on The Selected RTKs

Molecular docking simulations were carried out in four replicates on the Linux platform using AutodockVina® and associated tools after the validation of docking protocols. Binding free energy values (kcal/mol±SD) were ranked using an excel spreadsheet in order to identify the frontrunner phytochemicals.

Post-Docking Analysis

The Mean binding affinities and Standard Deviations were calculated and recorded on Microsoft Excel sheets. The binding affinities of the 68 phytochemicals were compared with that of the reference compounds. The phytochemicals affinities higher or equal to that of the reference compounds for each protein were collected and checked for drug likeness. While the phytochemicals with affinities lower than that of the reference compounds for each protein were screened out.

Drug-likeness and toxicity Assessment

The drug-likeness and toxicity assessment of the frontrunner phytochemicals was evaluated using data warrior software. Lipinski's rule of five was utilized to filter the frontrunners with drug-like properties. Frontrunner phytochemicals with no Lipinski violation were subjected to in-silico toxicity analysis. Data warrior assesses compounds for the following

toxicities: mutagenicity, tumorigenicity, reproductive effect and irritation effect. Frontrunners that showed any of this toxicity were deleted. Molinspiration was utilized to assess the bioactivity score of the phytochemicals for enzyme inhibitory effect. Swiss ADME was utilized to assess the bioavailability of the phytochemicals with multitargeting activity to determine their pharmacokinetic properties.

Table 2.1: Grid box parameters used in the molecular docking simulations

	EGFR- 8A27		**VEGFR- 6XVJ**		**FGFR- 5O49**	
	Centers	**Sizes**	**Centers**	**Sizes**	**Centers**	**Sizes**
X	**18.333**	**50**	**14.718**	**38**	**85.96**	**20**
Y	**-2.944**	**23**	**0.246**	**32**	**5.641**	**31**
Z	**-12.972**	**40**	**9.121**	**36**	**13.245**	**28**

3. RESULTS.

Qualitative Analysis Of *J.secunda* Leaf.

Table 3.1: Results for the presence of phytochemicals in the Ethanolic extract of *J.secunda*

Phytochemicals	**Results**
Alkaloids	+
Saponins	+
Tannins	+
Flavonoids	+
Steroids	+
Terpenoids	+
Cardiac glycosides	+

Carbohydrates	+
Proteins	+
Reducing Sugars	+

Molecular Docking of the Isolated *J. Secunda* Phytochemicals on the Selected RTKs

The molecular docking of phytochemicals was performed on the four proteins; EGFR, FGFR and VEGFR, to study and evaluate the ligand-receptor interactions at the binding sites of the proteins. For EGFR, the reference ligand had the highest binding energy to the receptor than the tested phytochemicals, but since molecular docking was carried out with other existing inhibitors (Gefitinib and Erlotinib), the 26 phytochemicals above Erlotinib were selected as frontrunner compounds. For FGFR, there were 25 frontrunner compounds with better binding energies than the reference ligand. For VEGFR, there were 10 compounds with higher binding energy than the reference compounds.

Table 3.3: Frontrunner compounds for EGFR, their mean binding energies and Standard deviation values.

S/N	NAME	MBE	STD
1	8A27 ligand	-10.40	1.40
2	Kaempferitrin	-9.63	0.26
3	Justiflorinol	-9.50	0.00
4	Pentanediyl bis-	-9.43	0.26
5	3_4_dihydroxyflavonol	-9.40	0.00
6	5H-Quinindoline	-9.40	0.00
7	Luteolin	-9.40	0.00
8	Cyclopentane-1,1'- [3-(2-cyclopentyl ethyl)-1,5-	-9.30	0.14
9	Apigenin	-9.30	0.00
10	Luteolin-7-O-rutinoside	-9.10	0.14
11	Diosmetin	-8.90	0.00
12	Squalene	-8.85	0.10
13	GefitinibEGFR	-8.70	0.41
14	Carinatone	-8.60	0.14
15	10H-Quinindoline	-8.50	0.00
16	Pentadecafluorooctanoic-acid-octadecyl-ester	-8.43	0.10
17	Roseoside	-8.38	0.05
18	Caffeoyl Glucoside	-8.23	0.17
19	Salicylic Glucoside	-8.20	0.12
20	Cyclohexane-1-(1,5-dimethylhexyl)-4-4-methylpentyl	-8.18	0.05
21	Hydroxy Jasmonic Acid Glucoside	-8.05	0.06
22	Juspurpurin	-8.05	0.06
23	Vitexin	-8.00	0.00
24	Cyclohexane-1-(cyclohexylmethyl)-4-ethyl-trans	-7.95	0.06

25	Vasicine	-7.90	0.00
26	Patentiflorin-A	-7.90	0.00
27	Erlotinib	-7.88	0.43

Table 4.5: Frontrunner compounds for VEGFR and their mean binding energies and standard deviation values

S/N	NAME	MBE	STD
1	Luteolin-7-O-rutinoside	-11.20	0.00
2	Diosmetin	-10.30	0.00
3	Apigenin	-10.20	0.00
4	Luteolin	-10.20	0.00
5	Cabozantinib	-10.15	0.51
6	3_4_dihydroxyflavonol	-10.10	0.00
7	5H-Quinindoline	-10.08	0.05
8	10H-Quinddoline	-9.60	0.00
9	Vatalanib	-9.43	1.12
10	Rutin	-9.38	0.05
11	6XVJLigand	-9.28	0.32

Table 3.5: Frontrunner compounds for FGFR and their mean binding energies and standard deviation

S/N	NAME	MBE	STD
1	Justicinol	-9.68	0.22
2	Jusmicranthin	-9.60	0.00
3	Patentiflorin-A	-9.55	0.06
4	TaiwaninE	-9.48	0.10
5	Procumbenoside-A	-9.40	0.12
6	PonatinibFGFR	-9.28	0.21
7	Juspurpurin	-9.03	0.15
8	Vitexin	-9.03	0.05
9	Luteolin-7-O-rutinoside	-8.85	0.24
10	ProcumbenosideA	-8.80	0.36
11	Elenoside	-8.70	0.00
12	Neojusticin-B	-8.70	0.00
13	Kaempferitrin	-8.70	0.00
14	CleistanthinB	-8.48	0.15
15	Justicidinoside-A	-8.30	0.08
16	Rutin	-8.28	0.05
17	3_4_dihydroxyflavonol	-8.20	0.00
18	Luteolin	-8.10	0.00
19	Diosmetin	-8.08	0.15
20	Justiflorinol	-7.90	0.00
21	Apigenin	-7.90	0.00
22	5H-Quinindoline	-7.88	0.05

23	10H-Quinindoline	-7.70	0.00
24	CilinaphthalideA	-7.68	0.22
25	LenvatinibFGFR	-7.65	0.13
26	5O49ligand	-7.50	0.08

4.4 Multi-targeting Phytochemicals against the RTKs studied

After the molecular docking, drug-likeness, and toxicity assessment, four compounds were selected as multitargeting compounds against the 3 RTKs studied.

These compounds include Luteolin, Diosmetin, 5H -Quindoline, and 10H-Quinindoline.

Table 3.7 shows the drug-likeness, toxicity, and bioactivity scores of the multi-targeting phytocompounds

s/n	Compound	MW dalton	cLogP	HA	HD	Muta—genicity	Tumori-genicity	Repro-ductive	Irritant	Kinase inhibitor
1	Diosmetin	300.27	2.26	6	3	None	None	None	None	0.25
2	Luteolin	286.24	1.99	6	4	None	None	None	None	0.26
3	5H-Quin doline	218.26	3.61	2	1	None	None	None	None	0.35
4	10H-Quindoline	218.26	3.45	2	1	None	None	None	None	0.10

Key; MW - Molecular weight.
HA- Hydrogen bond acceptors.
HD- Hydrogen bond donors.
cLogP- Octanol water coefficient.

4. DISCUSSION

The phytochemicals isolated and identified from the ethanolic/ethyl acetate leaf extract were subjected to in-silico molecular docking simulation on the receptor sites of these proteins. This was to determine the binding affinities of the identified phytochemicals to the four RTKs in comparison with reference compounds (co-crystallized with the receptor site on the protein) as well as already existing receptor tyrosine kinase inhibitors (Gefitinib, Erlotinib, Vatalanib, Cabozantinib, Tivantinib, Lenvatinib, Ponatinib). The frontrunner compounds were identified. These represent *J.secunda* phytochemicals that have better binding affinity to the receptors than the standard compounds as shown by having lower binding free energy values (kcal/mol). This means that theoretically, they can inhibit the RTKs to a much better degree than the existing inhibitors.

These compounds were subjected to further in-silico drug-likeness and toxicity testing and only 12 out of the 34 frontrunners passed the test.

The main interest was finding the multitargeting compounds, so we checked for compounds that had activity against the three RTKs, since HGFR did not have any frontrunners after molecular docking. Four compounds were seen to have multi targeting activity against EGFR, VEGFR and FGFR. They include; Luteolin, Diosmetin, 5H-quinindoline and 10H-Quindoline.

The biological activity of the phytochemicals was evaluated on the Molinspiration platform to obtain their bioactivity scores. The scores were categorized thus;

1. If the bioactivity score is between 0.00 to 0.50, the compound is considered active.
2. If the bioactivity score is between 0.00 to -0.50, the compound is considered moderately active.
3. If the bioactivity score is less than - 0.50, the compound is considered inactive.

The bioactivity scores show what class of proteins/enzymes the phytocompounds exert their biological activity on. The scores obtained show that the compounds are active receptor tyrosinase kinase inhibitors, according to the criteria above. These scores are seen in table 4.8 under the column, 'Kinase Inhibitor'.

The results obtained from this study show that *J.secunda* contain phytochemicals that have good potential anticancer properties via the inhibition of some RTKs that are necessary for the growth and proliferation of cancer cells. The compounds obtained from this study can be taken for further in-vitro and in-vivo testing to see if the same results will be obtained.

The in-silico approach helps shorten the duration and reduce the cost of drug discovery. It equally increases the likelihood of getting positive results in bioassays. Molecular docking is one of the quickest and most accurate in-silico methods for analyzing the molecular interactions and chemical bonding between a ligand and a protein.

This research successfully determined the multi-targeting *J.secunda* phytochemicals against the RTKs overexpressed in ovarian cancer. Further in-vitro studies should be carried out to confirm results.

5. CONCLUSION

In conclusion, this research successfully determined the multi-targeting *Justicia secunda* phytochemicals against ovarian cancer, using in-silico studies.

The study revealed four multi-targeting compounds; Luteolin, Diosmetin, 5H-Quindoline and 10H-Quindoline. These compounds showed promising in-silico ability to simultaneously inhibit the three Receptor tyrosine kinases (EGFR, VEGFR and FGFR) since HGFR did not have any multi-targeting compounds. This research by-passed the long, tasking and expensive process of testing all the isolated and identified phytochemicals in-vitro for this activity. By carrying out this research in-silico, we easily filtered out all the other phytocompounds that either failed drug-likeness tests or had some level of toxicity. This implies that the four compounds obtained can then be taken for further in-vitro testing to see if there is any benefit of testing its efficacy in-vivo, first in animal models and then in human patients.

The research provides valuable insights into the anticancer or enzyme inhibitory effects of *J.secunda* plant, thereby highlighting its potential use as a natural anticancer agent. However, it is important to acknowledge the limitations of this study, such as the fact that in-silico simulation may not fully mimic or represent the complex interactions that occur in living organisms especially in disease states like cancer.

5.2 RECOMMENDATIONS

To gain better insights into the potential anticancer properties of *J.secunda* leaves, we recommend that further in-vitro studies should be carried out using the ethanolic leaf extracts. This will help validate the results obtained from this study.

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