**In silico** approach to target PI3K/Akt/mTOR axis by selected *Olea europaea* phenols in PIK3CA mutant colorectal cancer

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**ABSTRACT**

Worldwide disease burden of colorectal cancer (CRC) increasing alarmingly, but a suitable therapeutic strategy is not available yet. Abnormal activation of the PI3K/Akt/mTOR signalling because of mutation in the PIK3CA gene is a driving force behind CRC development. Therefore, this study aimed to comprehensively characterise the potential of phenolic compounds from *Olea europaea* against the PI3K/Akt/mTOR axis by using *in silico* methodologies. Molecular docking was utilised to study key interactions between phenolic compounds of *O. europaea* and target proteins PI3K, Akt, mTOR with reference to known inhibitor of target. Drug likeness and ADME/T properties of selected phenols were explored by online tools. Dynamic properties and binding free energy of target-ligand interactions were studied by molecular dynamic simulation and MM-PBSA method respectively. Molecular docking revealed apigenin, luteolin, pinoreosinol, oleuropein, and oleuropein aglycone as the top five phenolic compounds which showed comparable/better binding affinity than the known inhibitor of the respective target protein. Drug likeness and ADME/T properties were employed to select the top three phenols namely, apigenin, luteolin, and pinoreosinol which shown to bind stably to the catalytic cleft of target proteins as confirmed by molecular dynamics simulations. Therefore, Apigenin, luteolin, and pinoreosinol have the potential to be used as the non-toxic alternative to synthetic chemical inhibitors generally used in CRC treatment as they can target PI3K/Akt/mTOR axis. Particularly, pinoreosinol showed great potential as dual PI3K/mTOR inhibitor. However, this study needs to be complemented with future *in vitro* and *in vivo* studies to provide an alternative way of CRC treatment.

**Abbreviations:** Akt: Protein kinase B; BBB: Blood-brain barrier; CNS: Central nervous system; CRC: Colorectal cancer; HBA: Hydrogen bond acceptor; HBD: Hydrogen bond donor; HIA: Human intestinal absorption; hERG: Human ether-a-go-go-related gene; MM-PBSA: Molecular mechanics Poisson-Boltzmann surface area; mTOR: mammalian target of rapamycin; MW: molecular weight; nROTB: number of rotatable bonds; OCT2: renal organic cation transporter-2; PDB: Protein data bank; PI3K: Phosphatidylinositol 3-kinase; RMSD: root means square deviation; RMSF: root mean square fluctuation; SASA: solvent accessible surface area; TPSA: total polar surface area; VDss: volume of distribution at steady state.

1. **Introduction**

Disease burden of colorectal cancer (CRC) is increasing rapidly all over the world because of environmental and dietary reasons along with the genetic factors (Yang et al., 2019). Over the years, CRC has emerged as one of the most predominant malignant tumours (Siegel et al., 2020). Scientific community is in a rush to find out the most suitable therapeutic strategy to combat colorectal carcinogenesis. Unfortunately, the current treatment strategy for CRC like surgery, chemotherapy and/or radiotherapy suffer from various undesirable side effects (Drott et al., 2018). Therefore, novel targeted therapy with minimised side effects is ever demanding for any type of cancer including CRC. Alterations in key signalling pathway components are common feature of all cancers. The signalling pathways including the phosphatidylinositol 3-kinase (PI3K), Akt (a serine/threonine kinase also known as protein kinase B), mammalian target of rapamycin (mTOR) signalling pathway is among the most important signalling pathways in the development and progression of CRC (Bahrami et al., 2018). Around 15–20% of CRC harbours activating mutations in the PIK3CA gene (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha) which affects the PI3Kα catalytic subunit (Park et al., 2019). Akt acts as the downstream effector of PI3K and regulates another downstream effector mTOR upon activation by phosphorylation (Xu et al., 2020). Overall, PI3K/Akt/mTOR axis is responsible for a range of
cellular activities related to carcinogenesis such as growth, proliferation, metabolism, angiogenesis, migration, and inhibition of apoptosis (Koveitypour et al., 2019). Therefore, PI3K/Akt/mTOR pathway has been emerged as a druggable target for CRC therapy. Several synthetic inhibitors (e.g. AZD8055, Tetraarsenic hexoxide) against this pathway has been tested in preclinical models of CRC (Chen et al., 2018; Nagappan et al., 2017). Unfortunately, these synthetic drugs often accompanied by several serious side effects like neutropenia, anaemia, thrombocytopenia, and diarrhoea which restrain their clinical use (Narayanankutty, 2019). On the other hand, phytochemicals, the non-nutritive secondary plant compounds are attaining popularity worldwide for their chemopreventive properties with no adverse side effects (Afrin et al., 2020). The olive trees (Olea europaea) are traditionally grown in Mediterranean basin. Both olive leaf as well as oil produced from olive fruit contain a varying concentration of phenolic compounds and known to have antioxidant and anti-cancer properties (Hashmi et al., 2015). Olive oil is an integral part of the Mediterranean cuisine and is linked to lower risk of developing colorectal cancer (Stoneham et al., 2000). Different types of phenolic compounds are abundant in olive oil including phenolic alcohols (tyrosol, hydroxytyrosol, etc.), secoiridoids (oleuropein, oleocanthal, etc.), and flavonoids (apigenin, luteolin, etc.). Many of the phenolic compounds shown promise as therapeutic intervention in various cancers including CRC by modulating different cellular signalling pathways crucial for cancer progression. Oleuropein, a phenolic secoiridoid from olive tree has been shown to downregulate PI3K/Akt and other CRC-related signalling pathways and thereby prevents colitis-associated CRC in pre-clinical animal model (Giner et al., 2016). Apigenin, a prominent flavonoid found in virgin olive oil has also been shown to inhibit cell proliferation and metastasis in CRC by reducing the phosphorylation of Akt (Chunhua et al., 2013). Another flavonoid from olive, luteolin can act as an anti-proliferative and pro-apoptotic agent in KRAS mutant human CRC cells, HCT115 by interacting with the PI3K/Akt pathway (Xavier et al., 2009). Different phenolic compounds from olive tree (e.g. hydroxytyrosol, oleuropein, oleocanthal) have also been shown to be effective against colorectal cancer in different in vitro and animal studies (Borzì et al., 2018). However, the precise molecular mechanisms of olive phenols in colorectal cancer is yet to be determined. In this context, we have studied the ability of phenolic compounds from Olea europaea to modulate the PI3K/Akt/mTOR axis by using in silico approaches with the hypothesis in mind that, these phenolic compounds might be better in modulating the PI3K/Akt/mTOR pathway in CRC with less or no side effects. Specific objectives of the present study were to reveal the possible interaction between phenolic compounds from Olea europaea and three key members of the PI3K/Akt/mTOR axis namely, PI3K, Akt, and mTOR to find out the best hits among these compounds by investigating the pharmacokinetic and toxicity profile for future drug development.

2. Methods

A total of 10 phenolic compounds from Olea europaea selected after literature review for this study and presence of these compounds in different parts of the olive tree was further verified from OliveNetTM Library (Table 1) (Bonvino et al., 2018).

2.1. Molecular docking studies

2.1.1. Protein preparations

The coordinates of the crystal structures PI3K, Akt, and mTOR were retrieved from the RCSB protein data bank (PDB) in pdb format. These crystal structures were cleaned prior to the docking by removing the bound ligands, ions, and water molecules. The crystal structures of PI3K, Akt, and mTOR were prepared for AutoDock Vina docking such as addition of polar hydrogens, charges, and assigning AD4 atom type. The preparation steps were carried out using AutoDock 4 (Morris et al., 2009).

2.1.2. Ligand preparations

Three-dimensional structure of each phenolic compound was retrieved from PubChem database in SDF (structure data file) format. The SDF format of ligand files was saved as PDB format using Marvin Sketch 19.2 and subsequently converted to pdbqt format using AutoDock 4. These pdbqt formats of ligand files were used for Vina docking.

2.1.3. Reference compounds preparation

The known inhibitors of PI3K, Akt, and mTOR available in RCSB protein data bank were used as reference compounds for olive phenolic compound screening. LY294002 (PubChem CID: 3973), GSX690693 (PubChem CID: 16725726), and pp242 (PubChem CID: 135565635) are the already reported inhibitors for PI3K, Akt and mTOR, respectively. The structures of reference compounds were retrieved from PubChem database as SDF format. The SDF format was then saved as PDB format using Marvin Sketch 19.2 and subsequently converted to pdbqt format for Vina docking using AutoDock 4.

2.1.4. Docking studies

Molecular docking was executed using AutoDock Vina 1.1.2. The grid box for docking was set in such a way that it can cover the whole protein. The compounds were screened based on Vina binding energy (Kcal/mol) and the interactions of ligands with target proteins were investigated with the help of PyMol 2.3 and Discovery Studio Visualizer v20.1.0.19195.

2.2. Drug likeness studies

The best five ligand molecules from Olea europaea based on molecular docking analysis were selected and examined utilising the Molinspiration Cheminformatics server (https://www.molinspiration.com/cgi-bin/properties). Drug-likeness parameters of the selected phenolic compounds were evaluated to check whether they comply with the Lipinski’s rule of five (e.g. molecular weight <500 g/mol, hydrogen bond donor <5, hydrogen bond acceptor <10). For each of the ligands, canonical SMILE molecular structure retrieved from the PubChem database (www.pubchem.ncbi.nlm.nih.gov) to analyse different parameters using the Molinspiration Cheminformatics server (Kim et al., 2019).
2.3. Absorption, distribution, metabolism, excretion, and toxicity (ADME/T) profile analysis

The in silico pharmacological activities of luteolin, apigenin, pinoresinol, oleuropein, and oleuropein aglycone were predicted based on their respective ADME/T profile. The ADME/T profile were explored by employing the pkCSM server (http://biosig.unimelb.edu.au/pkcsmprediction) (Pires et al., 2015). The pkCSM tool is useful to predict both pharmacokinetic properties of small molecules as well as toxicity by utilising graph-based signatures. The canonical SMILE molecular structures of individual ligands retrieved from the PubChem server (www.pubchem.ncbi.nlm.nih.gov) and utilised to reveal pharmacokinetic and pharmacodynamics parameters.

| Compound       | Source                        | PubChem CID | Structure | Reference                  |
|----------------|-------------------------------|-------------|-----------|----------------------------|
| Tyrosol        | Fruit, Leaf, Virgin oil, wastewater | 10393       | ![](image) | Je et al. (2015)           |
| Hydroxytyrosol | Fruit, Leaf, Virgin oil, wastewater | 82755       | ![](image) | Pei et al. (2016)          |
| Hydroxytyrosol acetate | Virgin oil | 155240     | ![](image) | Sánchez-Fidalgo et al. (2015) |
| Oleocanthal    | Virgin oil                    | 11652416    | ![](image) | Scotece et al. (2013)      |
| Oleuropein     | Fruit, virgin oil             | 5281544     | ![](image) | Liu et al. (2016)          |
| Oleuropein aglycone | Fruit, virgin oil            | 56842347    | ![](image) | Rigacci et al. (2015)      |
| Apigenin       | Virgin oil, wastewater        | 5280443     | ![](image) | Yang et al. (2018)         |
| Oleacein       | Leaf, virgin oil              | 18684078    | ![](image) | Polini et al. (2018)       |
| Pinoresinol    | Virgin oil, wastewater        | 73399       | ![](image) | Yu et al. (2019)           |
| Luteolin       | Fruit, Leaf, Virgin oil, wastewater | 5280445   | ![](image) | Yao et al. (2019)          |

2.4. Molecular dynamics simulations

Protein structures retrieved from the RCSB protein data bank were missing with some of the residues, which do not involved in binding to ligands; however, they may be involved the protein’s structural stability during simulation. So, prior to the MD simulation, we identified the missing residues of the PDB structures by comparing them with the UniProt sequence and modelled them using Modeller 10.1. Finally, the complex structures were made by redocking the modelled structure with selected ligands. Dynamics of phenolic compounds with target proteins were studied using GROMACS 2018.1 and GROMOS 54a7 force field. The complex of phenolic compound and target protein was solvated in a dodecahedron box with a distance of 1.0 nm from edge of the solvated box. The sodium (Na⁺) ions were added to neutralise the solvated system. Then the system was energy minimised with Lincs constraint-algorithm and steepest descent algorithm. The temperature and pressure of the systems were set to 300 K and one atmospheric pressure to mimic the general experiment conditions (Sabri et al., 2019). For the position restraining of ligands, ‘genrestr’ module was used. The temperature coupling groups was set to ‘tc-grps = Protein Non-Protein’ (Lemkul, 2019). LINCS algorithm, particle-mesh Ewald (PME), and Verlet algorithm were used for constraining bonds involving hydrogen, the electrostatic interactions, and the Van der Waals interactions, respectively.
Table 2. Summary of the top 5 scoring phenolic compounds against PI3K where LY294002 used as the reference compound for PI3K.

| Target protein | Phenolic compound | Binding energy (kcal/mol) | No. of H-bonds | Interacting amino acids | No. of non-covalent interactions | Interacting amino acids |
|----------------|-------------------|---------------------------|----------------|------------------------|----------------------------------|------------------------|
| PI3K (p110α)  | Luteolin          | -9.4                      | 2              | D810, V851              | 10                               | W780, I800, Y836, I848, I850, V851, M922, I932 |
| (PDB ID:5DXT) | Apigenin          | -9                        | 3              | V851, D933              | 8                                | I800, Y836, I848, V850, M922, I932 |
|                | Pinoresinol       | -8.3                      | 1              | S854                    | 10                               | W780, I800, I848, V850, R852, M922, I932 |
| Oleuropein glycone | -7.6              | 7                         | D810, V851, N428, S464, Q643, Q682, R683 | 3 | W446, P466, L645 |
| LY294002       | Oleuropein        | -7.2                      | 1              | N428                    | 1                                | M441 |
|                |                   | -8.7                      |                |                        | 5                                | Y836, I848, I932 |

(Lemkul, 2019). Short-range van der Waals cut off was set to 1.2 nm (Lemkul, 2019). The canonical ensemble of the complexes (NVT thermal equilibration) was performed using velocity-rescale thermostat specific to GROMACS for 100 ps with reference temperature of 300 K. Later the isobaric-isothermal ensemble (NPT pressure equilibration) was applied with the same temperature coupling. The Berendsen pressure coupling was used with reference pressure of one atmospheric pressure for 100 ps (Lemkul, 2019). Completely equilibrated system was subjected to the final molecular dynamics run for 100 ns by following same molecular dynamic procedure. The RMSD (root means square deviation), RMSF (root mean square fluctuation), hydrogen bonding, bond length between protein and ligand were analysed using GROMACS functions. MM-PBSA (Molecular Mechanics Poisson-Boltzmann Surface Area) binding energy of Protein-ligand complexes were calculated using g_mmpbsa GROMACS function (Kumari et al. 2014).

3. Results and discussion

3.1. Molecular docking study

As O. europaea is known to be a great source of various useful phytochemicals, the anti-cancer attributes of these phytochemicals are being tested vigorously all over the world (Castejón et al., 2020). In the current study, a set of 10 phenolic compounds from O. europaea namely, hydroxytyrosol, hydroxytyrosol acetate, oleocanthal, oleuropein, oleuropein aglycone, apigenin, tyrosol, pinoresinol, luteolin, and oleacine were used in molecular docking study against the three target proteins of PI3K-Akt-mTOR axis. For each of the target protein, known drug/inhibitor was used as a reference. Molecular docking revealed top five phenolic compounds for each of the target protein (Supplementary table 1). Results are further analysed below.

3.1.1. PI3K

Mutation in PIK3CA, which encodes the catalytic alpha subunit of PI3K (p110α), causes abnormally active PI3K in around 10–20% cases of colorectal cancer (Hamada et al., 2017). Herein, we observed that different phenolic compounds were able to bind with p110α [PDB ID: 5DXT], the catalytic subunit of class I PI3K at varying degree of strength. Among the ten olive phenols examined luteolin, apigenin, pinoresinol, oleuropein aglycone, and oleuropein displayed lowest binding energy; 9.4, −9, −8.3, −7.6, and −7.2 kcal/mol, respectively (Table 2). Two hydrogen bonds were formed by interaction of luteolin with p111α involving the residues Asp810 and Val851 at distances 2.18 Å and 2.22 Å, respectively (Figure 1(a)). The binding interaction was further stabilised via hydrophobic interactions involving amino acids Ile848, Tyr836, Ile800, Trp780, Val850, Val851, Met922, and Ile932 (listed in Table 2). On the other hand, apigenin showed three hydrogen bonding interactions (Val851 and Asp933) with 5DXT along with non-covalent interactions (Table 2 and Figure 1(b)). The interaction was further stabilised via one C-H bond (Ser774) (Figure 1(b)). Pinoresinol formed one H-bond (Ser854), one C-H bond (Glu798), and several other hydrophobic interactions (Table 2). O. aglycone and oleuropein interacted with the C2 and helical domain of p110α by forming H-bonds (7 H bonds in case of o. aglycone and 1Pi-donor H-bond in case of oleuropein) and hydrophobic interactions (Figure 1(d,e), and Table 2). LY294002, a known class I PI3K inhibitor (ATP-competitive inhibitor) used as a reference in molecular docking study (Xing et al., 2008). Luteolin and apigenin showed significantly less binding energy than LY294002 (−8.7 kcal/mol) while interacting with PI3K. So, it could be possible that both luteolin and apigenin have a strong affinity towards PI3K.

3.1.2. Akt

Akt acts as a suppressor of apoptosis machinery upon activation by PI3K (Nitulescu et al., 2018). Docking studies as shown in Table 3, suggests that luteolin, apigenin, pinoresinol, oleuropein, and o. aglycone were able to bind to Akt (PDB ID: 3QKK) with binding energy −8.1, −8, −8, −7.6, and −7.3 kcal/mol, respectively (Table 3). Four H-bonds were formed between luteolin and Akt involving three residues (Gly162, Phe161, and Glu191) (Figure 2(a)). Akt-luteolin interaction was further stabilised by 3 carbon-hydrogen bonds (Gly159 and Thr195) and 4 Pi-alkyl bonds (K179 and L181) (Figure 2(a)). Interaction between apigenin and 3QKK was stabilised by 3 H-bonds (Gly162 and Phe161), C-H bond (Gly159), and hydrophobic Pi-alkyl interactions (Table 3). Docking result further revealed that pinoresinol formed 2 H-bonds with residues Lys158 and Thr195 of 3QKK with an interatomic distance of 2.50Å and 2.51 Å respectively (Figure 2(c)). Pinoresinol also formed one carbon-hydrogen bond (Gly157) along with other hydrophobic interactions (Table 3).
Oleuropein was shown to form five H-bonds with 3QKK whereas o. aglycone able to form 4 H-bonds. GSK690693, an ATP competitive inhibitor of Akt1 used as a reference (Heerding et al., 2008), showed binding energy of \(-7.7\) kcal/mol while interacting with the target protein. The binding energy of the reference molecule is greater than the binding energy of luteolin, apigenin, and pinoresinol, indicating a strong binding affinity of these phenols to the Akt1 (Table 3).

Docking results further revealed that luteolin and apigenin interact with the catalytic Lys179 residue, required for ATP binding to the enzyme (Figure 2(a,b)). Therefore, luteolin and apigenin might interfere in Akt action by inhibiting the ATP binding to the enzyme.

### 3.1.3. mTOR

mTOR, a serine/threonine-protein kinase acts as a master regulator of cell growth and shown to be altered in various types of cancers including CRC. The mTOR protein is part of two structurally distinct signalling complex mTORC1 and mTORC2 (Stuttfeld et al., 2018). Here, we investigated the ATP-binding kinase domain of mTOR by using crystal structure of mTOR available at RCSB protein data bank [PDB ID: 4JT5]. Results of in silico binding experiments revealed that phenolic compounds from *O. europaea* bind to 4JT5 with varying degree of strengths. An estimated binding energy of \(-8.8\) kcal/mol of luteolin, making it the most effective phytochemical among the ten phenolic compounds tested (Table 4). Luteolin was able to form four H-bonds with three different amino acids (Asp2195, Asp225, Lys179), indicating a strong binding affinity of this compound to the mTOR protein.
Lys2187 and Val2240) and one Pi-donor H-bond (Asp2357) as demonstrated in Figure 3(a). Interaction between luteolin and 4JT5 was further stabilised by different types of hydrophobic interactions (pi-sulfur, pi-alkyl, etc.) (Table 4; Figure 3(a)). The binding energy of the apigenin-4JT5 complex was −8.4 kcal/mol with one H-bond with Val2240 at a distance of 1.95 Å, two Pi-donor H-bonds (Asp2357 and Trp2239), and hydrophobic interactions (Figure 3(b)). Similarly, strong binding ability of pinoresinol was also found with 4JT5 comparable to reference compound, pp242 (−8.9 kcal/mol), a known inhibitor of mTOR kinase (Table 4).

### 3.2. Drug likeness property

The best five phytochemicals for the target proteins were further analysed for various drug likeness parameters like molecular weight, number of hydrogen bond donor, number of hydrogen bond acceptor, etc. Top five phytochemicals namely, apigenin, luteolin, pinoresinol, oleuropein, and o. aglycone were found to have a logP (octanol/water partition

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**Table 4.** Summary of the top 5 scoring phenolic compounds against mTOR where pp242 used as the reference compound for mTOR.

| Target protein                  | Phenolic Compound | Binding energy (kcal/mol) | No. of H-bonds | Interacting amino acids | No. of non-covalent interactions | Interacting amino acids                  |
|--------------------------------|-------------------|---------------------------|----------------|-------------------------|----------------------------------|------------------------------------------|
| Serine/threonine-protein kinase | Luteolin          | −8.8                      | 5              | D2195, K2187, V2240, D2357 | 7                                | L2185, I2237, W2239, M2345, I2356         |
| mTOR [PDB ID-4JT5]             | Apigenin          | −8.4                      | 3              | V2240, W2239, D2357     | 9                                | L2185, I2237, W2239, M2345, I2356         |
|                                | Pinoresinol       | −8                        | 1              | D2357                  | 8                                | L2163, L2185, I2237, W2239, M2345, I2356 |
|                                | Oleuropein        | −6.9                      | 4              | Q1937, E2196           | 2                                | L1900, L1936                             |
|                                | G. aglycone       | −6.8                      | 4              | Q2142, R2224           | 4                                | L1936, A1971                             |
|                                | ppm242            | −8.9                      | 3              | D2195, V2225           | 14                               | L2185, I2237, W2239, M2345, I2356         |

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Figure 2. Three-dimensional and two-dimensional representation of interactions between Akt (3QKK) and ligands. (A) Akt/luteolin, (B) Akt-apigenin, (C) Akt-pinoresinol, (D) Akt-oleuropein, (E) Akt-o. aglycone, (F) Akt-GSK690693. Protein is depicted in grey surface, whereas ligands are portrayed as sticks.
coefficient) value less than 5, which suggest these compounds are likely to be absorbed effectively (Table 5). TPSA (total polar surface area) values for all these compounds barring oleuropein were less than 140 Å which indicate good Absorption Properties

Table 5. Drug-likeness property analysis of top 5 phenolic compounds from *O. europaea*.

| Compound Name       | MW (g/mol) (<500g/mol) | mLogP (<5) | HBA (<10) | HBD (<5) | nROTB (<10) | TPSA (Å²) | Lipinski Violation |
|---------------------|------------------------|------------|-----------|----------|-------------|-----------|-------------------|
| Luteolin            | 286.24                 | 1.97       | 6         | 4        | 1           | 111.12    | 0                 |
| Apigenin            | 270.24                 | 2.46       | 5         | 3        | 1           | 90.89     | 0                 |
| Pinoresinol         | 358.39                 | 2.59       | 6         | 2        | 4           | 77.39     | 0                 |
| Oleuropein          | 540.52                 | –0.36      | 13        | 6        | 11          | 201.68    | 3                 |
| Oleuropein aglycone | 378.38                 | 1.34       | 8         | 3        | 8           | 122.53    | 0                 |

Table 6. Predicted absorption properties of best-selected phenolic compounds.

| Properties                  | Luteolin | Apigenin | Pinoresinol | Oleuropein | Oleuropein aglycone |
|-----------------------------|----------|----------|-------------|------------|---------------------|
| Water solubility log mol/L  | –3.094   | –3.329   | –3.633      | –2.722     | –2.731              |
| Caco2 permeability (log 10 cm/s) | 0.096 | 1.007   | 1.036       | 0.067      | 0.61                |
| Human intestinal absorption (%) | 81.13 | 93.25   | 93.29       | 44.206     | 74.397              |
| Skin permeability log Kp    | –2.735   | –2.735   | –2.843      | –2.735     | –2.785              |
| P-glycoprotein substrate    | Yes      | Yes      | Yes         | Yes        | Yes                 |
| P-glycoprotein I inhibitor  | No       | No       | Yes         | No         | No                  |
| P-glycoprotein II inhibitor | No       | No       | No          | No         | Yes                 |

Table 7. Predicted distribution properties of best-selected phenolic compounds.

| Properties       | Luteolin | Apigenin | Pinoresinol | Oleuropein | Oleuropein aglycone |
|------------------|----------|----------|-------------|------------|---------------------|
| VDs (log L/kg)   | 1.153    | 0.822    | 0.036       | 0.54       | 0.241               |
| Fraction unbound (human) (Fu) | 0.168 | 0.147 | 0          | 0.485      | 0.373               |
| BBB permeability (log BB) | –0.907 | –0.734 | –0.439      | –1.921     | –1.309              |
| CNS permeability (log PS) | –2.251 | –2.061 | –2.975      | –4.735     | –3.375              |
absorption capacity of these compounds (Table 5). Another topological parameter checked was nROT (number of rotatable bonds), which measures molecular flexibility and is related to oral bioavailability of drugs (Veber et al., 2002). All five phenolic compounds were flexible as apigenin and luteolin have 1 rotatable bond, pinoresinol has 4 rotatable bonds, o. aglycone has 8 and oleuropein has 11 rotatable bonds (Table 5). Except oleuropein, which violated 3 properties, all other compounds luteolin, apigenin, pinoresinol, and o. aglycone exhibited no violation and followed ‘Rule of 5’ (Table 5). Therefore, oleuropein was excluded from further analysis.

### 3.3. ADME/T profile

In addition to physicochemical efficacy of a compound to inhibit a target protein, several other parameters like absorption, distribution, metabolism, excretion, and toxicity of the compound should be investigated to reveal the odds of success of a drug candidate. Therefore, we executed detailed ADME/T profiling of the best five phenolic compounds as revealed by molecular docking along with the reference compounds. Permeability to colorectal adenocarcinoma cell line, Caco-2 and human intestinal absorption are considered as two crucial parameters to predict the bioavailability of a candidate drug. Among tested compounds (luteolin, apigenin, pinoresinol, oleuropein, and o. aglycone), apigenin and pinoresinol showed high Caco-2 permeability potential (>8 × 10^{-6} cm/s), the other three showed comparatively low Caco-2 permeability potential (<8 × 10^{-6} cm/s); and all of them could be absorbed through human intestine (predicted HIA value >30%) (Table 6) (Pires et al., 2015). All the five compounds were predicted as the substrates of permeability P-glycoprotein (Table 6), an ATP-binding cassette transporter which acts as an efflux membrane transporter, abundant in epithelial cells. However, pinoresinol was also predicted as a P-glycoprotein I inhibitor, and oleuropein aglycone as a P-glycoprotein II inhibitor (Table 6). Therefore, pinoresinol and o. aglycone could modulate the physiological roles of P-glycoprotein.

Volume of distribution at steady-state (VDss), an important parameter related the distribution of drugs in plasma or tissue was also checked under ADME/T profile. Higher the VDss value, greater the chance of distribution of drugs in tissue rather than plasma (Pires et al., 2015). As predicted by the pkCSM server among the five tested compounds, pinoresinol displayed the least value required for uniform distribution in the plasma. Luteolin showed the highest value of VDss, suggesting its distribution in tissues. Another crucial parameter, the ability of degree of diffusion across plasma membrane of each phenolic compounds were checked as well. The result indicates the increase in the following order pinoresinol < apigenin < luteolin < o. aglycone < oleuropein, which was measured as the fraction present in the unbound state (Table 7). It is also revealed these five phenolic compounds have very limited access to the brain and are incompetent to penetrate the central nervous system; implying no damaging effect on CNS.

Cytochromes P450 (CYP) isozymes, mainly found in liver act as the principal detoxification enzyme of the body. As revealed by the pkCSM server, when tested, pinoresinol and o. aglycone come out as substrate of CYP3A4, predicted to be effectively metabolised by CYP3A4. It was also revealed that luteolin is a CYP1A2 and CYP2C9 inhibitor; apigenin is a CYP1A2 and CYP2C19 inhibitor; pinoresinol is a CYP1A2, CYP2C19 and CYP3A4 inhibitor (Table 8). These data are particularly important as inhibition or activation of CYP enzymes results in clinically relevant drug-drug interactions and may precede unexpected adverse reactions or even therapeutic failures (Lynch & Price, 2007).

Drug clearance is another important parameter which is linked to the bioavailability of a drug and vital to determine dosing rates to attain steady-state concentrations (Pires et al., 2015). None of the tested compounds were predicted as substrate of renal OCT2 transporter (renal organic cation transporter-2) as presented in the Table 9. This result suggests tested compounds are perhaps cleared through alternative routes like bile, faeces, sweat etc. to avoid interactions with any other drugs administered. Pinoresinol was having the least value of total clearance, whereas, oleuropein has the maximum value (Table 9).

Next, we did the toxicological assessments of luteolin, apigenin, pinoresinol, oleuropein, and o. aglycone. Except o. aglycone, none of the compounds were associated with hepatotoxicity as predicted by the pkCSM server. Therefore, o. aglycone was excluded from further studies. AMES test results revealed that tested compounds could be non-carcinogenic (Table 10). None of the tested compounds shown to inhibit the human ether-a-go-go-related gene I (hERG I) which translates a subunit of the voltage-gated Kv11.1 potassium ion channel. Drug interaction with this channel may lead to reduced channel function and acquired long QT syndrome (Sanguinetti, 2014). Thus, tested compounds might not block potassium channels, however oleuropein was revealed as hERG II inhibitor. It was also revealed that none
of these compounds is associated with skin sensitisation and hence, most probably not induce allergic contact dermatitis.

3.4. Molecular dynamics simulation

After screening of phenolic compounds from the *O. europaea* through different tools such molecular docking, drug-likeness filter, and ADME/T profiling, we have shortlisted the three most suitable candidates, apigenin, luteolin, and pinoresinol, as the top three inhibitors for all three target proteins (PI3K/Akt/mTOR). The molecular dynamic simulation has been further utilised to assess their suitability as a drug candidate. The parameters used for simulations were the same for all protein-ligand complexes. The dynamic behaviours of ligands were compared with reference ligands reported as inhibitors of target proteins.

3.4.1. Root mean square deviation and root mean square fluctuation

The spatial variations of target proteins during molecular dynamics simulation upon binding of ligands were analysed by measuring the root means square deviation (RMSD) of the protein backbone and root mean square fluctuation (RMSF) of each residue. The backbone of PI3K (p110α) complexes stabilised between 0.2 nm and 0.4 nm after five ns of simulation. However, the backbone of the PI3K-apigenin complex starts deviating between 0.4 nm to 0.6 nm after 30 ns of simulation (Figure 4). After 60th ns, all the complexes were stabilised below the PI3K (protein alone) except the PI3K-apigenin complex. The reduced variations in complexes indicate that the interaction of drug candidates with PI3K was more stable, and it did not affect the structure of PI3K backbone (Banavath et al., 2014). Root mean square fluctuation of complexes and apo-PI3K were calculated against each residue of PI3K over 100 ns simulation (Figure 5).

The measurement of the spatial variations at the residual level (RMSF) gave the details about the residues which are more sensitive to drug binding. Residue numbers 785 to 795 and 990 to 1020 of PI3K located outside of the ligand-binding sites deviated more than other residues upon binding of pinoresinol and luteolin, respectively (Figure 5). The mean RMSF of all PI3K-phenolic compounds was lower than the apo-PI3K, which means the drug candidates can form a stable and strong complex with the active site of PI3K (Table 11).

In the case of Akt, the backbone of protein-ligand complexes and apo-Akt was stabilised between 0.2 nm and 0.3 nm after 2 ns of simulation (Figure 4). More deviation observed in the Akt-luteolin complex up to 20th ns; after that, all the graphs were mostly overlapped; however, the minor deviation was observed in complexes. The mean value of RMSD was calculated by taking an average of 5000 frames (every 10 picoseconds) for all complexes and apoproteins.

The mean RMSD of complexes was slightly higher than apo-Akt (Table 11). The residual level fluctuations of Akt upon phenolic compound binding helped to identify the complexes stabilised between 0.2 nm and 0.4 nm after five ns of simulation. However, the backbone of the PI3K-apigenin complex starts deviating between 0.4 nm to 0.6 nm after 30 ns of simulation (Figure 4). After 60th ns, all the complexes were stabilised below the PI3K (protein alone) except the PI3K-apigenin complex. The reduced variations in complexes indicate that the interaction of drug candidates with PI3K was more stable, and it did not affect the structure of PI3K backbone (Banavath et al., 2014). Root mean square fluctuation of complexes and apo-PI3K were calculated against each residue of PI3K over 100 ns simulation (Figure 5).

The measurement of the spatial variations at the residual level (RMSF) gave the details about the residues which are more sensitive to drug binding. Residue numbers 785 to 795 and 990 to 1020 of PI3K located outside of the ligand-binding sites deviated more than other residues upon binding of pinoresinol and luteolin, respectively (Figure 5). The mean RMSF of all PI3K-phenolic compounds was lower than the apo-PI3K, which means the drug candidates can form a stable and strong complex with the active site of PI3K (Table 11).

In the case of Akt, the backbone of protein-ligand complexes and apo-Akt was stabilised between 0.2 nm and 0.3 nm after 2 ns of simulation (Figure 4). More deviation observed in the Akt-luteolin complex up to 20th ns; after that, all the graphs were mostly overlapped; however, the minor deviation was observed in complexes. The mean value of RMSD was calculated by taking an average of 5000 frames (every 10 picoseconds) for all complexes and apoproteins.

The mean RMSD of complexes was slightly higher than apo-Akt (Table 11). The residual level fluctuations of Akt upon phenolic compound binding helped to identify the

### Table 10. Predicted toxicological profile of best-selected phenolic compounds.

| Compound          | Ames Toxicity | hERG I Inhibitor | hERG II Inhibitor | Hepatotoxicity | Skin Sensitivity |
|-------------------|---------------|------------------|------------------|----------------|-----------------|
| Luteolin          | No            | No               | No               | No             | No              |
| Apigenin          | No            | No               | No               | Yes            | No              |
| Pinoresinol       | No            | No               | Yes              | No             | No              |
| Oleuropein        | No            | No               | No               | Yes            | No              |
| Oleuropein aglycone | No        | No               | No               | No             | No              |

Figure 4. Root-mean-square deviation (RMSD) of the backbone of protein-ligand complexes were plotted over 100 ns MD simulation. The colour code of the respective ligand-target complexes was mentioned at the top of the figure. LY294002, GSK690693, and pp242 are the control ligand for PI3K, Akt, and mTOR, respectively.
residues responsible for the slight deviations in structure. Residue number 180 to 190, which are loop region outside of the binding site, caused the deviation in the backbone of the Akt-luteolin complex; similarly, 345 to 365 and 415 to 422 for Akt-pinoresinol complex, and 226 to 250 and 423 to 460 for control inhibitor caused the deviation in the backbone of Akt (Figure 5). The mean RMSF of Akt-ligand complexes was lower than the apo-Akt (without ligand) (Table 11). It shows that the binding of these three drug candidates to Akt can stabilise the active site residues of Akt, which are involved in interaction and form stable complexes in each case. The RMSD of mTOR backbone of all complexes and apo form stabilise around 0.4 nm after 40 ns simulation (Figure 4). After 70th ns, the deviation of complexes was lesser than the apo-mTOR, and the overall deviation of mTOR complexes is relatively lower than the apo-mTOR (Table 11). The binding of drug candidates to the mTOR causes spatial variations in some of the mTOR non-active site residues. RMSF analysis, which gives insight about the residual fluctuations at the non-active site upon binding of drug candidates, shown the residual fluctuations in residue numbers 2271 to 2286, 2367 to 2396, and 2425 to 2436 for apigenin, control, and pinoresinol complex, respectively. In the case of the mTOR-luteolin complex, minor deviations were observed at residue numbers 2254 to 2261. The binding site residues involved in the interactions were stabilised upon binding of drug candidates (Figure 5). So, it can be inferred that these drug candidates can form a stable complex with the kinase domain of mTOR.

### 3.4.2. H-Bond analysis

The strength of the interaction between protein and drug candidates was further analysed by measuring the number of H-bond and length of H-bond throughout the simulation.

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**Table 11.** Mean values of molecular dynamics simulation parameters.

|                | PI3K only | PI3K-Apigenin | PI3K-Luteolin | PI3K-Pinoresinol | PI3K-LY294002 |
|----------------|-----------|---------------|---------------|------------------|---------------|
| RMSD (nm)      | 0.3149    | 0.3801        | 0.2752        | 0.3087           | 0.2671        |
| RMSF (nm)      | 0.1803    | 0.1654        | 0.1629        | 0.1648           | 0.1578        |
| H-Bonds        | 2.18      | 2.54          | 1.38          | 1.2              |               |
| Pair distance (nm) | 0.1795 | 0.1794        | 0.1914        | 0.1914           | 0.2346        |

|                | Akt only | Akt-Apigenin | Akt-Luteolin  | Akt-Pinoresinol  | Akt-GSK690693 |
|----------------|----------|-------------|---------------|------------------|--------------|
| RMSD (nm)      | 0.2832   | 0.2849      | 0.2971        | 0.3021           |              |
| RMSF (nm)      | 0.1928   | 0.1693      | 0.1839        | 0.1689           | 0.1767       |
| H-Bonds        | 2.58     | 2.46        | 2.06          | 0.80             |              |
| Pair distance (nm) | 0.1776 | 0.1858      | 0.1807        | 0.2384           |              |

|                | mTOR only | mTOR-Apigenin | mTOR-Luteolin | mTOR-Pinoresinol | mTOR-pp242   |
|----------------|-----------|---------------|---------------|------------------|--------------|
| RMSD (nm)      | 0.4090    | 0.3851        | 0.3902        | 0.3824           | 0.3474       |
| RMSF (nm)      | 0.2180    | 0.2203        | 0.1892        | 0.2044           | 0.1755       |
| H-Bonds        | 1.44      | 2.1          | 1.26          | 1.07             |              |
| Pair distance (nm) | 0.1938 | 0.1904       | 0.1839        | 0.1956           |              |

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*Figure 5. Root mean square fluctuation (RMSF) of protein-ligand complexes at the residual level. The colour code of the respective target-ligand complexes was mentioned at the top of the figure. LY294002, GSK690693, and pp242 are the control ligand for PI3K, Akt, and mTOR, respectively.*
H-bond formed between target proteins and drug candidates during 100 ns simulation were varied from 0 to 8 (Figure 6).

The mean number of H-bonds formed between target proteins and drug candidates was greater than the control ligands (Table 11). The strength of the H-bond formed was also analysed by the pair distance between donor and acceptor. Jefferies categorised H-bonds with a donor-acceptor distance of 2.2–2.4 Å as strong, 2.5–3.2 Å as moderate, and 3.2–4 Å as weak (Dannenberg, 1998). The mean pair distance of all target protein-ligand complexes was less than 2.4 Å (0.24 nm) (Figure 7 and Table 11).

We have also analysed the instability of H-bonds by monitoring the binding pose of drug candidates. The protein-drug complex structures were retrieved from the trajectory with 20 ns interval and superimposed with 0th ns to monitor the binding pose of drug candidates. Positional changing of

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**Figure 6.** A plot is showing inter-molecular hydrogen bonds between target proteins and drug candidates over 100 ns MD simulation. The colour code of the respective target-ligand complexes was mentioned at the top of the figure. LY294002, GSK690693, and pp242 are the control ligand for PI3K, Akt, and mTOR, respectively.

**Figure 7.** Hydrogen bond pair distance between donor and acceptor of protein-ligand complexes over 100 ns MD simulation. The colour code of the respective ligand-target complexes was mentioned at the top of the figure. LY294002, GSK690693, and pp242 are the control ligands for PI3K, Akt, and mTOR, respectively.
drug candidates within the binding pocket was observed for all the complexes, and the drug candidates remained in the same binding pocket (Figures 8–10).

In the Akt-GSK690693 complex (control for Akt), GSK690693 moved from its original binding pocket to another after 20th ns and remained in contact with Akt (Figure 9). These results confirmed that the number of H-bonds formed between target proteins and drug candidates was good enough to hold the drug inside the binding pockets.
3.4.3. Binding free energy of complexes

The lifetime of drug candidates presents inside the binding pocket is determined by the cumulative of electrostatic and non-electrostatic interactions (Erbas et al., 2018). We have calculated the binding free energy of each complex with 10 ns intervals up to 100 ns using the Molecular Mechanics Poisson-Boltzmann Surface Area (MMPBSA) method. The MMPBSA method is frequently used for the calculation of binding free energy between small molecules and proteins (Wang et al., 2018). The binding free energy of protein-drug candidate complexes in an aqueous solvent was calculated using the formula described previously (Wang et al., 2018). The calculated binding free energy of all protein-drug candidate complexes shown in Table 12.

GSK690693 (reference molecule for Akt) showed better binding energy than all complexes irrespective of target proteins. We have also noticed that for all the complexes, non-electrostatic energy contributed more to the binding free energy than electrostatic energy, and it is vice versa for the Akt-GSK690693 complex (Table 12). All three phenolic compounds (apigenin, luteolin, and pinoresinol) showed a higher value of binding energy against Akt1 (Table 12) when compared to GSK690693. Therefore, it could be possible that these phytochemicals are weak inhibitors of Akt1. Apigenin and pinoresinol showed better affinity (binding energy $-95.348$ KJ/mol and $-73.433$ KJ/mol, respectively) against the target protein mTOR when compared to the reference molecule (pp242), luteolin and pinoresinol showed comparably

![Figure 10. Dynamics of top 3 phenolic compounds and control (pp242) on mTOR (4JT5) surface during molecular dynamics simulation (0th-100th ns). Each phenolic compound is denoted in six different colours based on simulation time, red-0th ns, green-20th ns, blue-40th ns, magenta-60th ns, cyan-80th ns, and orange-100th ns.](image)

| Table 12. Predicted binding free energy of all protein-ligand complexes by MMPBSA method. |
|---------------------------------------------------------------|
| PI3K-ligand | Van der Waal energy (KJ/mol) | Electrostatic energy (KJ/mol) | Polar solvation energy (KJ/mol) | SASA energy (KJ/mol) | Binding energy (KJ/mol) |
| Apigenin | $-140.386$ | $-57.680$ | 145.535 | $-16.219$ | $-68.750$ |
| Luteolin | $-149.005$ | $-48.392$ | 127.670 | $-16.238$ | $-85.965$ |
| Pinoresinol | $-145.100$ | $-42.047$ | 131.927 | $-17.918$ | $-73.137$ |
| LY2940042 | $-142.222$ | $-4.208$ | 49.467 | $-14.449$ | $-109.412$ |

| Akt-ligand | Van der Waal energy (KJ/mol) | Electrostatic energy (KJ/mol) | Polar solvation energy (KJ/mol) | SASA energy (KJ/mol) | Binding energy (KJ/mol) |
| Apigenin | $-140.816$ | $-55.369$ | 137.101 | $-14.658$ | $-73.742$ |
| Luteolin | $-99.1118$ | $-77.954$ | 125.337 | $-13.464$ | $-24.898$ |
| Pinoresinol | $-163.007$ | $-44.179$ | 129.412 | $-16.880$ | $-94.655$ |
| GSK690693 | $-115.070$ | $-342.092$ | 296.168 | $-14.363$ | $-175.338$ |

| mTOR-ligand | Van der Waal energy (KJ/mol) | Electrostatic energy (KJ/mol) | Polar solvation energy (KJ/mol) | SASA energy (KJ/mol) | Binding energy (KJ/mol) |
| Apigenin | $-151.045$ | $-27.056$ | 97.463 | $-14.710$ | $-95.348$ |
| Luteolin | $-136.007$ | $-74.526$ | 180.383 | $-14.730$ | $-44.880$ |
| Pinoresinol | $-141.102$ | $-32.123$ | 115.201 | $-15.490$ | $-73.433$ |
| pp242 | $-117.794$ | $-26.478$ | 91.673 | $-12.547$ | $-65.146$ |
strong affinity (binding energy $-85.965 \text{ KJ/mol}$ and $-73.137 \text{ KJ/mol}$, respectively) towards PI3K. Pinoresinol showed better binding energy ($-94.655 \text{ KJ/mol}$) against Akt than the other two compounds although, the binding affinity is much lower than the reference molecule (Table 12). Therefore, it is possible that luteolin, pinoresinol, and apigenin interfere within the PI3K/Akt/mTOR axis.

4. Conclusion

The PI3K/Akt/mTOR pathway is frequently altered in cancer, and activation of this pathway due to the mutation in the PIK3CA gene is found in around 20% of metastatic CRC (Foley et al., 2017). In our study, 10 phenolic compounds present in Olea europaea were investigated to uncover their anti-cancerous attributes against the components of the selected pathway. Binding affinities of five phenolic compounds (apigenin, luteolin, pinoresinol, oleuropein, and o. aglycone) towards all three target proteins (PI3K, Akt, and mTOR) showed similar or even better magnitude, compared to the known inhibitor of individual protein as revealed by AutoDock Vina docking studies.

Most of the selected compounds were able to form H-bond and other non-covalent interactions with the catalytic cleft of the target proteins. Drug likeness and ADME/T filters were applied to find out the most suitable drug candidates and to rule out any unwanted toxicity. MD simulation further justified the selection of the top three phenolic compounds (apigenin, luteolin, and pinoresinol) as those are bound with target proteins right through the simulation period (Figures 4–10). Binding free energy as calculated by MMPBSA method revealed that luteolin is the best ligand among olive phenolic compounds tested against PI3K (p110$\alpha$) and pinoresinol binds to the PI3K with good binding affinity as well. Most importantly, the binding pose from the molecular docking study revealed that the interaction between PI3K and luteolin or pinoresinol is similar to the interaction between PI3K and ATP-competitive inhibitor of PI3K (LY294002) by interacting with the same residues. Although none of our selected phenols able to interact with Akt with the same intensity as the reference molecule (GSK690693), pinoresinol is the best molecule to bind with Akt than the rest of the phenols present in O. europaea. Apigenin and pinoresinol interacted with the mTOR kinase domain residues (aa 2182-2516) and have a stronger binding affinity even than the second-generation ATP-competitive inhibitor of mTOR (pp242) revealed by the MMPBSA analysis. Therefore, collectively all these results suggest that apigenin, luteolin, and pinoresinol could be useful to restrain the PI3K/Akt/mTOR axis in particular PIK3CA mutant colorectal cancer. Another noteworthy finding from our study is that pinoresinol may serve as the best alternative among the phenolic compounds tested to the synthetic
dual PI3K/mTOR inhibitors currently being used as therapeu tic intervention. To our best knowledge, this is the first study to characterise the interaction between pinoresinol and major components of PI3K/Akt/mTOR axis, PI3K and mTOR. This will particularly help in current CRC drug development. Drug likeness analysis and toxicity profiling of the phenolic compounds further strengthen our findings. Overall findings of this study are summarised in Figure 11.

Based on our current study, it can be concluded that phenolic compounds from *O. europaea* are potent modulators of PI3K/Akt/mTOR axis to combat CRC. However, further research is obligatory in order to evaluate the efficacy of selected phytochemicals in the treatment of CRC.

**Disclosure statement**

Authors declares no competing interest.

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**Authors’ contributions**

A.S. conceived and planned the manuscript with D.N.; A.S. performed the literature review. A.S. and T.K. performed experiments. T.K. analysed the molecular docking and molecular dynamic simulations results. A.S. critically edited manuscript. All authors read and approved the final manuscript.

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