A network pharmacology-based approach to explore potential targets of *Caesalpinia pulcherima*: an updated prototype in drug discovery

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*Caesalpinia pulcherima* (CP) is a traditional herb used for the treatment of asthma, bronchitis, cancer, anti-bacterial, anti-fungal and as abortifacient. In the present study, bioactive components and potential targets in the treatment of breast cancer validated through in silico, in vitro and in vivo approach. The results for the analysis were as among 29 components, only four components were found active for further study which proved the use of CP as a multi-target herb for betterment of clinical uses. The results found by PPI states that our network has significant interactions which include the ESR-1, ESR-2, ESRR, MET, VEGF, FGF, PI3K, PDK-1, MAPK, PLK-1, NEK-2, and GRK. Compound-target network involves 4 active compound and 150 target genes which elucidate the mechanisms of drug action in breast cancer treatment. Furthermore, on the basis of the above results the important proteins were fetched for the docking study which helps in predicting the possible interaction between components and targets. The results of the western blotting showed that CP regulates ER and EGFR expression in MCF-7 cell. In addition to this animal experimentation showed that CP significantly improved immunohistological status in MNU induced carcinoma rats. Network pharmacology approach not only helps us to confirm the study of the chosen target but also gave an idea of compound-target network as well as pathways associated to the CP for treating the complex metabolic condition as breast cancer and they importance for experimental verification.
research and development for TCM herbs or herbal formulae. It provides a new logical guide and technical route for developing and understanding TCM drugs’ mechanisms of action. NP encourages the discovery of successful molecules, recognizing their interrelationship, elucidating the relationship between TCM formulae and diseases or TCM syndrome, establishing logical TCM drugs, as well as directing integrated use of TCM and conventional drugs\(^\text{11-13}\). CP decoction or infusion traditionally used as purgative, tonic in the treatment of convulsions, intermittent fevers, lung and skin diseases, cure bad cough, breathing difficulty and chest pain, treats inflammations, earache, muscular and rheumatic pain and various cardiovascular disease, cancer and other chronic diseases\(^\text{14,15}\).

In our previous study we have tried to investigate the effect of ethyl acetate fraction of CP in breast cancer rats and possible molecular mechanism involved by the CP\(^\text{16}\). Therefore, this study extends to use NP approach to establish the effect of CP in BC and to predict core targets and biological functions, pathways and mechanism of action. Molecular docking was also performed on selected active components and the key targets to validate the NP results.

### Results

#### Components in CP.

According to literature and TCMID, TCM, TCMSP databases 61 active components were found in CP. The selected components were further subjected to evaluate pharmacokinetics parameters i.e. ADME criteria for OB ≥ 30% and DL ≥ 0.18 values are shown in Table 1.

| MOL ID     | Components       | OB%  | DL%  | BBB  |
|------------|------------------|------|------|------|
| MOL000513  | Gallic acid      | 31.69| 0.04 | −0.54|
| MOL000889  | Catechin         | 54.83| 0.24 | −0.73|
| MOL00415   | Rutin            | 3.2  | 0.68 | −2.75|
| MOL01002   | Ellagic acid     | 43.06| 0.43 | −1.41|
| MOL00098   | Quercetin        | 46.43| 0.28 | −0.77|
| MOL01111   | α-Pinene         | 46.25| 0.05 | 2.18 |
| MOL01111   | β-Pinene         | 44.77| 0.05 | 2.29 |
| MOL00023   | Limonene         | 39.84| 0.02 | 2.12 |
| MOL01115   | E-Verbeneol      | 50.68| 0.06 | 1.48 |
| MOL00232   | α-Terpineol      | 48.8 | 0.03 | 1.72 |
| MOL007510  | α-Copaene        | 37.81| 0.08 | 2.04 |
| MOL00036   | E-Caryophyllene  | 29.7 | 0.09 | 2.07 |
| MOL001177  | β-Copaene        | 29.47| 0.12 | 2.04 |
| MOL001180  | α-Muurolene      | 21.53| 0.08 | 2.05 |
| MOL001123  | β-Muurolene      | 19.5 | 0.08 | 2.16 |
| MOL004724  | γ-Cadinene       | 21.34| 0.08 | 2.09 |
| MOL012609  | E-Nerolidol      | 29.61| 0.06 | 1.44 |
| MOL01323   | 1-Epi-Cubenol    | 58.49| 0.09 | 1.49 |
| MOL001123  | α-Phellandrene   | 19.5 | 0.08 | 2.16 |
| MOL00911   | α-Terpineol      | 33.95| 0.02 | 2.1  |
| MOL00257   | β-Phellandrene   | 56.28| 0.12 | 2.06 |
| MOL00122   | 1,8-Cineole      | 43.75| 0.05 | 2.27 |
| MOL000202  | γ-Terpineol      | 33.02| 0.02 | 2.05 |
| MOL000920  | Linalool         | 49.37| 0.04 | 0.74 |
| MOL012618  | Trans-linalool oxide (pyranoid) | 22.91| 0.07 | 1.07 |
| MOL00608   | Terpinen-4-ol    | 32.16| 0.03 | 1.52 |
| MOL00036   | β-Caryophyllene  | 29.7 | 0.09 | 2.07 |
| MOL001193  | Caryophyllene oxide | 32.67| 0.13 | 1.76 |
| MOL008549  | Cyanidin 3-glucoside | 58.99| 0.24 | −0.04 |

Table 1. The specific information of anti-breast cancer components in *Caesalpinia Pulcherima*.

### Screening of components of CP for breast cancer.

Among the 61 components, only 29 components were found to relate with breast cancer as shown in Table 1. Using TCMSP the 29 components were subjected to OB and DL criteria filtering out with only four active components of CP based on the ADME criteria. The genes of each active component were fetched from Swiss target prediction whereas genes of the disease i.e., breast cancer were retrieved from GeneCards Human database.

### Construction and analysis of target PPI network.

In order to enhance the visualization and understand the mechanism of the targets, it is important to study the PPI of the target genes. The target genes of the corresponding components were subjected to STRING v_11 to visualize and construct the PPI network.
for the same. The high-confidence target protein interaction data was set with a score level greater than 0.9. The interactions between the target proteins is depicted in Fig. 1, which comprises of total 124 nodes, 233 edges; each edge represents PPIs. The other parameter is average node degree which values at 3.76 and local clustering co-efficient: 0.538 corresponds to the number of targets that are connected to the network. Degree plays an important role in showing the role of proteins interaction and nodes of network. The PPI network shows the targets involved in breast cancer are ESR-1, ESR-2, ESRRA, MET, VEGF, FGF, PI3K, PDK-1, MAPK, PLK-1, NEK-2, and GRK which are the major targets in breast cancer. Along with ESR-1; EGFR, MET, and VEGFR are located centrally in the network which indicates the role of proteins in the pathogenesis of breast cancer. Basically, ESR pathway is a forthcoming starting point to discover the mechanisms of breast cancer. The main factor in ESR pathway is estrogen which is found to be an integral component involved in the development and maturation of breast. The involvement of estrogen receptors are also seen in other pathological processes including breast cancer, endometrial cancer, and osteoporosis. So, PPI network and pathway analysis of novel genes were carried out for the recognition of critical genes related to the breast cancer.

**Arrangement and construction of disease-target network.** To study the signaling pathway and function of the selected target genes, the data was imported to Cytoscape to construct compound-target network.
In Fig. 2, the compound-target-disease interaction network was constructed which elucidates the mechanisms of drug action in the breast cancer treatment. It consists of 4 ingredients, and 150 interactive target proteins. In this network, we found that many targets were hit by multiple components. This fact inferred that the active biochemical of CP might influence these targets synergistically; it has therapeutic effects on other disease and disorders additionally to BC. The details of three topological parameters i.e. Betweenness Centrality, Closeness Centrality, and Degree are given in Table 2 which gives an important role of each target in the network structure.

**GO gene enrichment analysis and KEGG pathway annotation.** GO enrichment analysis was carried out to analyse the target proteins. The setting for the ClueGO was set for three criteria to analyse the target genes for GO biological (Table 3), Go molecular (Table 4), and GO cellular (Table 5) and the most important parameter as KEGG pathway (Fig. 3A). The GO term fusion was restricted to pV ≤ 0.005 that is based on the false discovery rate (Benjamini-Hochberg). The Ras-Raf-MAPK signaling pathway is a key route for the ErbB family, as is the PI3K/AKT pathway, both of which result in alteration of cell proliferation and apoptosis. As far as breast cancer is considered, over-expression of ErbB receptor may lead to Ras activation. So to analyse the statement, GO and KEGG analysis was carried to determine the signaling pathway and following pathways were found to associate: PI3K-Akt, MAPK, ErbB, Ras, Chemokine, HIF-1, FoxO, sphingolipid, AMPK, VEGF, JAK-STAT, TGF, insulin, GnRH, estrogen signaling pathway, prolactin signaling pathway, thyroid hormone signaling pathway, and relaxin signaling pathway (Fig. 3B).

CP can be used for the treatment of other conditions also as hepatitis B, measles, human T-cell leukemia virus 1 infection, Kaposi sarcoma-associated herpes virus infection. As the constituents of CP was selected to examine the effects on breast cancer but via KEGG analysis (Table 6) it was found responsible in many cancers as colorectal cancer, renal cell carcinoma, pancreatic cancer, endometrial cancer, prostate cancer, melanoma, bladder cancer, small cell lung cancer, non-small cell lung cancer, hepatocellular carcinoma, and gastric cancer. Owing to the facts and visualization CP may be used as novel drug for the treatment of various diseases and disorder.

**Molecular modelling: docking of the active components.** After analysing the pathways and diseases and disorder related to the genes, it is important to study structure based design of the component, as well as its ability to predict the binding-conformation of small molecule ligands to the appropriate target binding site. The
| Name    | Betweenness centrality | Closeness centrality | Clustering coefficient | Degree | Topological coefficient |
|---------|------------------------|----------------------|------------------------|--------|------------------------|
| AKT1    | 0.15105568             | 0.42124542           | 0.23913043             | 24     | 0.16908213             |
| GSK3B   | 0.00818                | 0.3422619            | 0.33333333             | 7      | 0.28011204             |
| CDK1    | 0.04618775             | 0.3133515            | 0.42222222             | 10     | 0.25                   |
| CCNA2   | 0.05253746             | 0.32951289           | 0.48888889             | 10     | 0.23658537             |
| CSNK2B  | 0                     | 0.22157996           | 1                      | 2      | 0.59090909             |
| CSNK2A1 | 0.01619132             | 0.24364407           | 0.16666667             | 4      | 0.27777778             |
| VEGFA   | 0.05497117             | 0.40636042           | 0.31481481             | 28     | 0.18434874             |
| KDR     | 0.00489856             | 0.40636042           | 0.27272727             | 23     | 0.1970547              |
| TOP2A   | 0.03747492             | 0.26995305           | 0.44444444             | 9      | 0.32716049             |
| IL5     | 0.00137232             | 0.35060976           | 0.73333333             | 6      | 0.38461538             |
| IL2     | 0.06628375             | 0.36741214           | 0.58333333             | 9      | 0.3132969              |
| CCNA1   | 0.01018686             | 0.3042328            | 0.7                    | 5      | 0.39230769             |
| PIK3R1  | 0.11353431             | 0.40209793           | 0.24070474             | 28     | 0.17176871             |
| INSR    | 0.172E–04              | 0.33141213           | 0.6                    | 9      | 0.2251462              |
| IGF1R   | 0.0013309              | 0.36257311           | 0.58333333             | 9      | 0.38164251             |
| NEK2    | 0                     | 0.26436782           | 1                      | 6      | 0.49019608             |
| CREB1   | 0.05089584             | 0.37337662           | 0.31818181             | 12     | 0.2295082              |
| FLT4    | 1.24E–04               | 0.32122905           | 0.80952381             | 7      | 0.42857143             |
| AGL     | 0                     | 1                     | 0                      | 1      | 0                      |
| PYGL    | 0                     | 1                     | 0                      | 1      | 0                      |
| ESR1    | 0.05830291             | 0.3938562            | 0.36263736             | 14     | 0.21978022             |
| CCND1   | 0.16791787             | 0.41071429           | 0.33333333             | 18     | 0.20425355             |
| MMP9    | 0.08713433             | 0.39655172           | 0.375                  | 16     | 0.2240635              |
| PLK4    | 0                     | 0.26436782           | 0.7                    | 6      | 0.49019608             |
| MAPT    | 0.00399207             | 0.30831099           | 0.3                    | 5      | 0.30344828             |
| TERT    | 0.02200057             | 0.35276979           | 0.52308952             | 7      | 0.35428571             |
| PFG     | 0.0063316              | 0.35276074           | 0.5                    | 12     | 0.29716981             |
| TPT1    | 0                     | 0.23662551           | 0                     | 1      | 0                      |
| FGF1    | 0.00111343             | 0.33823529           | 0.63636364             | 11     | 0.34042553             |
| FGF2    | 0.07499146             | 0.3938562            | 0.3762338             | 22     | 0.21306818             |
| MMP2    | 0.00461767             | 0.35714286           | 0.57777778             | 10     | 0.29423077             |
| MET     | 6.53E–04               | 0.34124629           | 0.77777778             | 9      | 0.4                    |
| SMAD3   | 0.00192975             | 0.33625731           | 0.46666667             | 6      | 0.3484848              |
| ALOX5   | 6.10E–05               | 0.21821632           | 0.9047619             | 7      | 0.77777778             |
| PTGS1   | 0.09914773             | 0.26932084           | 0.67857143             | 8      | 0.50961538             |
| FLT3    | 0.00238706             | 0.33045977           | 0.66666667             | 7      | 0.41883117             |
| TKE     | 3.95E–04               | 0.33045977           | 0.71428571             | 7      | 0.44480519             |
| ESR2    | 0.11758808             | 0.37828947           | 0.47619048             | 7      | 0.30295567             |
| LYN     | 0.0306857              | 0.36741214           | 0.45454545             | 31     | 0.24633431             |
| SYK     | 0.00326702             | 0.33045977           | 0.61904762             | 7      | 0.35191638             |
| HSD17B1 | 0                     | 0.23760331           | 1                      | 3      | 0.66666667             |
| HSD17B2 | 0                     | 0.23760331           | 1                      | 3      | 0.66666667             |
| B4GALT1 | 0.5                   | 0.8                   | 0.33333333             | 3      | 0.55555556             |
| ST3GAL3 | 0                     | 0.66666667           | 1                      | 2      | 0.75                   |
| FGR     | 0.01597583             | 0.34638554           | 0.46428571             | 8      | 0.25510204             |
| ST6GAL1 | 0                     | 0.5                   | 0                      | 1      | 0                      |
| PTK2    | 0.0039192              | 0.34534353           | 0.51515152             | 12     | 0.30065359             |
| MMP3    | 3.67E–04               | 0.33823529           | 0.86666667             | 6      | 0.37037037             |
| MPG     | 0.02074822             | 0.29187817           | 0.6                    | 2      | 0.5                    |
| APEX1   | 0.0201823              | 0.24678112           | 0                      | 4      | 0.25                   |

Continued
| Name       | Betweenness centrality | Closeness centrality | Clustering coefficient | Degree | Topological coefficient |
|------------|------------------------|----------------------|------------------------|--------|-------------------------|
| IL6        | 0.21357151             | 0.42279412           | 0.22134387             | 23     | 0.16205534              |
| ALOX15     | 0.00360723             | 0.22373541           | 0.75                   | 8      | 0.60227273              |
| ALOX12     | 6.10E−05               | 0.21821632           | 0.9047619              | 7      | 0.77777777              |
| ARG1       | 0.03662179             | 0.32951289           | 0.4                    | 6      | 0.27222222              |
| POLJ       | 0.0173913              | 0.22373541           | 0                      | 2      | 0.5                     |
| POLJH      | 0                      | 0.18312102           | 0                      | 1      | 0                       |
| MAPK8      | 0                      | 0.29715762           | 0                      | 1      | 0                       |
| CYP1B1     | 0.11543811             | 0.30263158           | 0.3                    | 5      | 0.28571429              |
| GUSB       | 0.04549909             | 0.27058824           | 0.5                    | 4      | 0.45                    |
| MPO        | 0.13589779             | 0.33823529           | 0.4                    | 6      | 0.27027027              |
| ALOX15B    | 0                      | 0.21780303           | 1                      | 6      | 0.83333333              |
| AXL        | 0                      | 0.29262087           | 1                      | 2      | 0.65517241              |
| ABCG1      | 0                      | 0.32046092           | 0                      | 1      | 0                       |
| ABCG2      | 0.0173913              | 0.2987013            | 0                      | 2      | 0.5                     |
| ESRRB      | 0                      | 0.28822055           | 1                      | 2      | 0.72222222              |
| SNCA       | 0.24232927             | 0.29113924           | 0.23809524             | 7      | 0.28571429              |
| CXCR1      | 0.0825549              | 0.35527697           | 0.3055556              | 9      | 0.21604938              |
| CAMK2B     | 0.00167032             | 0.32122905           | 0.16666667             | 4      | 0.35810811              |
| F2         | 0.00591872             | 0.35493827           | 0.3                    | 5      | 0.35686275              |
| ALDH1B1    | 0.01815372             | 0.20282187           | 0.16666667             | 4      | 0.2857143              |
| AKR1A1     | 0.03250421             | 0.22373541           | 0                      | 3      | 0.38888889              |
| CYP1C9     | 0.00360723             | 0.22373541           | 0.75                   | 8      | 0.60227273              |
| CYP1A2     | 0.03517144             | 0.24416136           | 0.3                    | 5      | 0.45                    |
| AKR1B1     | 0.00833383             | 0.18341308           | 0.2                    | 5      | 0.4                     |
| QDPR       | 0.02290496             | 0.20390071           | 0.33333333             | 3      | 0.45833333              |
| AVPR2      | 0.0173913              | 0.29113924           | 0                      | 2      | 0.5                     |
| CYP2C8     | 0.00280628             | 0.22300997           | 0.80952381             | 7      | 0.63636364              |
| PIM1       | 0                      | 0.28822055           | 1                      | 2      | 0.61111111              |
| EPHB4      | 3.05E−04               | 0.32030371           | 0.33333333             | 3      | 0.47747748              |
| OPD1       | 0                      | 0.27251185           | 1                      | 5      | 0.50769231              |
| DRD4       | 0.0465845              | 0.27777778           | 0.66666667             | 6      | 0.4047619               |
| PARG       | 0                      | 0.19827586           | 0                      | 1      | 0                       |
| P4HB       | 0                      | 0.29792746           | 0                      | 1      | 0                       |
| MAOA       | 0.03380981             | 0.22637795           | 0                      | 3      | 0.357                  |
| ADORA1     | 0.00744264             | 0.27380952           | 0.66666667             | 6      | 0.42307692              |
| TTR        | 0.00942748             | 0.31944444           | 0.83333333             | 4      | 0.36111111              |
| MMP13      | 4.88E−05               | 0.32485876           | 0.93333333             | 6      | 0.42682927              |
| KLK2       | 0.00705145             | 0.29262087           | 0                      | 2      | 0.5                     |
| FUT4       | 0.5                    | 0.8                  | 0.33333333             | 3      | 0.55555556              |
| PKN1       | 0.00167811             | 0.25054466           | 0                      | 2      | 0.5                     |
| NMR2       | 0.04100187             | 0.32030371           | 0.52380952             | 7      | 0.2409015               |
| AKR1B10    | 0.00183066             | 0.18282989           | 0.66666667             | 3      | 0.5                     |
| HSD17B8    | 0.03606286             | 0.24159664           | 0.5                    | 4      | 0.46428571              |
| POLD3      | 0.00607311             | 0.25842697           | 0                      | 2      | 0.5                     |
| XDH        | 0                      | 0.1965812            | 0                      | 1      | 0                       |
| CCR4       | 0                      | 0.27251185           | 1                      | 5      | 0.50769231              |
| ALPL       | 0                      | 1                    | 0                      | 1      | 0                       |
| ALPI       | 0                      | 1                    | 0                      | 1      | 0                       |
| NEU1       | 0                      | 0.24838013           | 0                      | 1      | 0                       |
| IAPP       | 0                      | 0.2259332            | 0                      | 1      | 0                       |
| DYRK1A     | 0.001214               | 0.28255528           | 0.33333333             | 3      | 0.45098039              |
| ELAVL1     | 0.00120149             | 0.31944444           | 0.66666667             | 4      | 0.39102564              |
| ALK        | 0                      | 0.31081081           | 1                      | 2      | 0.77142857              |
| PLA2G1B    | 0                      | 0.2173913            | 1                      | 5      | 0.84444444              |
| GLO1       | 0.00350393             | 0.19166667           | 0                      | 2      | 0.6                     |
| ARPP19     | 0                      | 0.23908524           | 0                      | 1      | 0                       |
| Continued  |                        |                      |                        |        |                         |
The main reason behind selecting the proteins is that they played a major role in PPI, Compound-target network and in KEGG analysis; as well as these proteins are found to play a vital role in the mechanism of BC. Table 7 and Fig. 4A–E gives the detail about docking score and poses of active components i.e., cyanidin, catechin, ellagic acid, and quercetin against c-MET (PDB Id: 5EYD), EGFR (PDB Id: 4ZAU), PDGFR-α/β (PDB Id: 5GRN), VEGFR (PDB Id: 2OH4), and ERR (PDB Id: 1ERR-α).

### Table 2. Important nodes with network analyzer results.

| Name     | Betweenness centrality | Closeness centrality | Clustering coefficient | Degree | Topological coefficient |
|----------|------------------------|----------------------|------------------------|--------|-------------------------|
| FUT7     | 0                      | 0.5                  | 0                      | 1      | 0                       |
| NOX4     | 7.19E−05               | 0.30503979           | 0.66666667             | 3      | 0.51851852              |
| BACE1    | 0.0344775              | 0.28606965           | 0                      | 2      | 0.5                     |
| GRK6     | 0                      | 0.25164114           | 0                      | 1      | 0                       |
| CBR1     | 0.0027995              | 0.18821604           | 0                      | 2      | 0.6                     |
| FASN     | 0                      | 0.29715762           | 0                      | 1      | 0                       |
| ADORA2A  | 0.01137633             | 0.2804878            | 0                      | 2      | 0.5                     |
| NT5E     | 0.00173277             | 0.23279352           | 0                      | 2      | 0.5                     |
| ARCB1    | 0                      | 0.28967254           | 0                      | 1      | 0                       |
| CA9      | 0                      | 0.30585106           | 1                      | 2      | 0.70833333              |
| SLC5A1   | 0                      | 0.28967254           | 0                      | 1      | 0                       |
| HSPA1A   | 0                      | 0.29792746           | 0                      | 1      | 0                       |
| PON1     | 0                      | 0.29792746           | 0                      | 1      | 0                       |

### Table 3. GO biological process.

| Description                                    | Count in gene set | False discovery rate |
|-----------------------------------------------|-------------------|----------------------|
| Organic substance metabolic process           | 112               | 4.42 × 10−21         |
| Response to organic substance                 | 67                | 4.42 × 10−21         |
| Metabolic process                             | 113               | 1.65 × 10−20         |
| Cellular response to chemical stimulus        | 64                | 1.90 × 10−20         |
| Phosphate-containing compound metabolic process| 57                | 2.01 × 10−20         |
| Cellular metabolic process                    | 108               | 1.09 × 10−19         |
| Protein phosphorylation                        | 40                | 1.09 × 10−19         |
| Response to chemical                          | 76                | 3.80 × 10−19         |
| Regulation of cell death                      | 49                | 1.84e × 10−19        |
| Organonitrogen compound metabolic process     | 84                | 2.00 × 10−18         |

### Table 4. GO molecular function.

| Description                                    | Count in gene set | False discovery rate |
|-----------------------------------------------|-------------------|----------------------|
| Catalytic activity                            | 100               | 4.23 × 10−20         |
| Protein kinase activity                       | 35                | 3.07 × 10−20         |
| Phosphotransferase activity, alcohol group as acceptor | 37                | 7.44 × 10−20         |
| Transferase activity, transferring phosphorus-containing groups | 40                | 2.82 × 10−19         |
| Kinase activity                               | 37                | 7.10 × 10−19         |
| Ion binding                                   | 86                | 3.07 × 10−18         |
| Protein tyrosine kinase activity              | 19                | 2.31 × 10−15         |
| Anion binding                                 | 55                | 1.68 × 10−14         |
| Drug binding                                  | 42                | 5.65 × 10−13         |
| Transferase activity                          | 48                | 5.69 × 10−13         |

**In vitro experimental validation.** The KEGG analysis indicated that PI3K-Akt, Ras-Raf-MAPK and estrogen signaling pathway are a key route associated with breast cancer treatment by CP. To verify the reliability of obtained targets in network pharmacology analysis, we have conducted western blot analysis for ER and EGFR protein expression to confirm signaling pathway in breast cancer effects of the CP. The expression of ER
and EGFR were declined after 24 h in MCF-7 cells treated with EAFCP at dose level 200 µg/ml. The obtained results indicate that EAFCP may suppress estrogen regulated EGFR mediated signaling pathway (Fig. 5).

**In vivo experimental validation.** In this part to reveal the molecular mechanism of EAFCP (ethyl acetate fraction of *Caesalpinia pulcherima*) in treating MNU (N-Methyl-N-nitrosourea) induced mammary carcinoma, the anti-tumour effect improved after treatment for 30 days. EAFCP 500 mg/kg and TAM (Tamoxifen) 2 mg/kg tumour-bearing rats were significantly more resistant to the development tumour than control rats and observed decreased tumour development (Fig. 6A–C). Consequently, we have examined the effect of treatment on the density of ER-α expression by immunohistochemistry (Fig. 7A–D). After EAFCP and TAM treatment, ER-α immunoreactivity inside the nucleus was reduced significantly. The results strongly established that the treatment worsen tumour development by interfering ER.

**Discussion**

As far as traditional approach is considered “one drug, one target” theory of drug design is used, in contradictory network pharmacology which aims to explore the correlation of drugs and diseases, based on the multi-targeted therapy17. Novelty of this approach includes the use of systems biology, network analysis, connectivity, and redundancy. NP studies was successfully used to identify the novel targets and to determine the unknown signaling pathways interact with compounds18,19. The NP approach provides new insights into the systemic connection between therapeutic targets, and a disease as a whole and provides a powerful and promising tool for the clarification of disease mechanisms at a systemic level and the discovery of potential bioactive ingredients20.

In this context the present study generated a novel network which gives a general view of molecular mechanism of CP.

Active components found in CP are ellagic acid, gallic acid, cyanidine, catechin, quercetin, rutin, β-sitosterol, myricetin, flavonoids, homo-flavonoids, pulcherrimin, lupeol, glycosides and phenols. The components were screened for its DL and OB criteria and thus, four active components i.e. quercetin, ellagic acid, cyanidine, and catechin were found suitable for further studies. BC network constructed through the plant bioactive target followed by recognition of targets associated with BC pathway. The network reveals the potential of 4 CP bioactives to modulate the BC by the interactions of 150 proteins through multiple pathways. These components inhibit cells proliferation, induces cell cycle arrest and apoptosis in different cancer cell types21–25. Through PPI interaction of the genes we found 124 nodes and 233 edges. ESR-1, ESR-2, ESRR-A, MET, FGF, VEGF, PI3K, PDK-1, MAPK, PLK-1, NEK-2, and GRK were likely to be key genes in the development of BC. GO and KEGG analysis revealed several pathways as well as other disease and disorders for the selected genes. The GO enrichment analysis showed the direct involvement of bioactive in the regulation of BC. KEGG pathway analysis proved that estrogen signaling pathway and ErbB signaling pathway may be crucial signaling pathway in the selected network which helps to support that CP may be used for BC treatment. Other than estrogen signaling pathway and ErbB, Ras, Chemokine, HIF-1, FoxO, sphingolipid, PI3K-Akt, AMPK, VEGF, JAK-STAT, TBF, insulin signaling pathway, GnRH, estrogen signaling pathway, prolactin signaling pathway, thyroid hormone signaling pathway, and relaxin signaling pathway were also found, suggesting the use of CP in multi-targets. Adding on the evidence for CP it can be used in colorectal cancer, renal cell carcinoma, pancreatic cancer, endometrial cancer, prostate cancer, melanoma, bladder cancer, small cell lung cancer, non-small cell lung cancer, hepatocellular carcinoma and gastric cancer. In addition to this, the docking study was carried for the validation of targets. It also screens the affinity between the components and targets, which can directly clarify their structure–activity relationship. On the basis of the results obtained in network pharmacology, therapeutic effect of CP was investigated by western blotting, signifying that EAFCP treatment could regulates ER signaling pathway and suggesting that breast cancer can be treated through a complex system with multi-component target disease interaction. Our earlier preclinical study on the phytochemicals from CP decrease cell proliferation and induce apoptosis, significantly improved the pathological conditions of MNU induced breast cancer rat tissue suggesting their involvement in BC treatment16. It is not yet known whether giving CP alone or with

| Description                  | Count in gene set | False discovery rate |
|------------------------------|-------------------|----------------------|
| Extracellular region         | 45                | 1.29 × 10⁻⁰⁸         |
| Cytoplasmic part             | 93                | 1.71 × 10⁻⁰⁷         |
| Cell periphery               | 65                | 2.31 × 10⁻⁰⁷         |
| Plasma membrane              | 63                | 6.99 × 10⁻⁰⁷         |
| Cytoplasm                    | 100               | 3.59 × 10⁻⁰⁶         |
| Plasma membrane part         | 39                | 1.79 × 10⁻⁰⁶         |
| Membrane                     | 81                | 3.08 × 10⁻⁰⁶         |
| Intracellular organelle lumen| 58                | 5.02 × 10⁻⁰⁶         |
| Cytosol                      | 56                | 5.64 × 10⁻⁰⁶         |
| Cell part                    | 120               | 6.81 × 10⁻⁰⁶         |

Table 5. GO cellular component.
Figure 3. (A) Kyoto encyclopaedia of genes and genomes pathway (Contributing co-author: Shweta A. More). (B) Gene ontology enrichment analysis (contributing co-author: Shweta A. More).
A chemotherapeutic agent will enhance the activity in treating patients with breast cancer which is future scope of the present study.

**Conclusion**

This study scientifically investigates the pharmacological mechanism of CP in the treatment of breast cancer through network pharmacology, docking analysis, western blotting and in vivo animal study. It is in addition worth mentioning that network pharmacology has great advantages in clearing up the mechanism of CP as TCM.

**Materials and methods**

The following parameters are important in order to construct a network: (1) identification and confirmation of compounds using chemical databases; (2) selection of compounds on the basis of pharmacokinetic parameter i.e. ADME (absorption, distribution, metabolism, and excretion) criteria; (3) selected compounds were further subjected to understand protein interaction and to obtain the relevant information by using publicly available database or tools; (4) genes related to target disease i.e., breast cancer were identified using human disease database and common genes of target and compounds were selected; (5) construction and analysis were carried out to understand the interaction and molecular mechanisms using visualization software; and (6) to perform docking study of the active constituents.

**Chemical databases.** The chemical components of CP were identified through literature and the Traditional Chinese Medicine Ingredient Database (TCMID, https://www.megabionet.org/tcmid/); the TCM Database@Taiwan (https://tcn.cmu.edu.tw/), most comprehensive databases on global scale. The chemical components were subjected to database called Traditional Chinese Medicine Systems Pharmacology (TCMSP, https://lsp.nwu.edu.cn/tcmsp.php) to screen for breast cancer. TCMSP helps to promote integration of both traditional as well as modern medicines in order to accelerate the drug discovery which builds the framework for system pharmacology and covers the ADME information. Chemical structures, synonyms, molecular weight, canonical SMILES and physicochemical properties were collected with the help of ChEMBL (https://www.ebi.ac.uk) and Pubchem (https://pubchem.ncbi.nlm.nih.gov).

**Evaluation of pharmacokinetics parameters.** The selected components were further screened for oral bioavailability and drug-likeness pharmacokinetics parameters which include ADME. The ADME characteristics of the drug indicate the ratio of the oral drug to oral dosage of the blood circulatory system. The parameters to access the components druggability is analysed according to the set parameters as oral bioavailability (OB ≥ 30) value and drug-likeness (DL ≥ 0.18) indices recommended by TCMSP. Among all the selected components, only the components which fit in the criteria were selected for construction of network.

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### Table 6. KEGG pathways.

| Description                                      | Count in gene set | False discovery rate |
|--------------------------------------------------|-------------------|----------------------|
| Pathways in cancer                               | 28                | 1.08 × 10^-15        |
| PDK-Akt signaling pathway                        | 24                | 1.11 × 10^-15        |
| EGFR tyrosine kinase inhibitor resistance        | 12                | 3.19 × 10^-11        |
| Metabolic pathways                               | 33                | 1.13 × 10^-10        |
| Ras signaling pathway                            | 16                | 1.46 × 10^-10        |
| Proteoglycans in cancer                          | 15                | 1.78 × 10^-10        |
| MAPK signaling pathway                           | 17                | 3.66 × 10^-10        |
| Endocrine resistance                             | 11                | 1.93 × 10^-09        |
| AGE-RAGE signaling pathway in diabetic complications | 11        | 2.33 × 10^-09        |
| Rap1 signaling pathway                           | 14                | 2.33 × 10^-09        |

### Table 7. Docking score of tyrosine kinase (interacting amino acid). Bold signifies that among each class of tyrosine kinase the maximum score of the active constituent.

| Active constituents | Docking Score of tyrosine kinase (interacting amino acid) |
|---------------------|----------------------------------------------------------|
|                     | c-Met (PDB ID: 5EYD) | EGFR (PDB ID: 4ZAU) | PDGFR α/β (PDB ID: 5GRN) | VEGFR family (PDB ID: 2OH4) | ERR (PDB ID: 1ERR-α) |
| Catechin            | − 6.6651 (TYR 1230) | − 7.969 (GLU 762)   | − 10.392 (PHE 837)      | − 7.793 (CYS 917)    | − 9.533 (MET 421)   |
| Cyaniding           | − 7.315 (ARG 1208)  | − 7.747 (GLU 762)   | − 10.302 (GLU 644)      | − 11.233 (LEU 838)  | − 8.995 (LEU 346)   |
| Ellagic acid        | − 7.348 (GLY 1163)  | − 5.792 (MET 793)   | − 4.396 (VAL 815)       | − 4.727 (ARG 1025)  | − 8.197 (LEU 346)   |
| Quercetin           | − 7.576 (ALA 1221)  | − 7.417 (GLU 762)   | − 10.056 (LEU 599)      | − 10.224 (CYS 917)  | − 9.378 (GLU 353)   |
Identification of target genes and construction of target PPI (protein–protein interaction) network. PPI is important aspect to study the involvement of proteins in various biochemical processes in order to understand the cellular organization, bioprocess and functions. This can be done by using the virtual screening database called STRING 11.0 (https://string-db.org/)\(^\text{31}\). The genes of the selected components were uploaded to STRING to get the information about PPIs. The setting for generating the PPI network was in accordance with ‘Homo sapiens’ and the confidence in the interaction among the target protein was set to the highest confidence data > 0.9. The network nodes represent proteins whereas the edge represents associated protein–protein.

Identification of disease target genes. In order to construct the compound-target network, it is important to identify the genes related to disease. The information related to breast cancer associated target genes was collected from GeneCards\(^\text{32}\) (https://www.genecards.org), human gene database which provides information related to all annotated and predicted human genes.

Construction of compound-target network. Once the protein–protein interaction was carried, the next step is to understand the molecular mechanism which is achieved by constructing the compound-target network using Cytoscape\(^\text{33}\) visualization software v_3.7.1. The compound-target network helps to understand and analyse the mechanism of the components with target as well as the pathway involved.

Figure 4. Docking analysis of components and targets. (A) Catechin and EGFR (GLU 762). (B) Catechin and FGFR (PHE 837). (C) Cyanidin and 1ERR (LEU 346). (D) Cyanidin and VEGFR (LEU 838). (E) Quercetin and c-Met (ALA 1221) (contributing author: Santosh N Mokale).
Gene Ontology (GO) gene enrichment analysis and Kyoto encyclopaedia of genes and genomes (KEGG) pathway annotation. GO and KEGG pathway annotation is carried on ClueGO, another Cytoscape plug-in which gives a network-based visualization to diminish redundancy of results from pathway enrichment analysis. GO is carried out to analyse the gene cluster in the network to improvise the data prediction. GO provides a hierarchically organized set of thousands of standardized terms for biological processes, molecular functions and cellular components, with curated and predicted gene annotations based on these terms for multiple species. Biological process GO annotation is frequently used resource for pathway enrichment analysis. The study objective is to identify the biological process in order to layout the meaningful functional information. KEGG is used to study gene functions and the metabolic pathway of the inputted network of genes and molecules. It also helps us to find out the contributing pathway of the target associated with the disease.

Molecular modelling: docking of the active components. Docking study was carried out to find the affinity as well as orientation of the selected active component by docking them against the selected receptors as c-MET, EGFR, PDGFR-α/β, VEGF, and ERR using Glide v_7.6 program interfaced with Maestro v_11.3 of Schrödinger 2017 (Schrödinger, LLC, New York, NY, USA). The crystal structure for c-MET (PDB Id: 5EYD), EGFR (PDB Id: 4ZAU), PDGFR-α/β (PDB Id: 5GRN), VEGFR (PDB Id: 2OH4), and ERR (PDB Id: 1ERR-α) were taken from RCSB Protein Data Bank and prepared for docking using protein preparation wizard. The structures of compounds were built using Maestro build panel and optimized to lower energy conformers using Ligprep v_3.3.
Experimental validation

Western blot analysis. The cell lysates from EAFCP (200 µg/ml) treated MCF-7 cells were obtained and protein concentrations were measured using a Bradford protein assay kit (Bio-Rad, USA). Approximately, 50 µg lysate was resolved on 10% SDS-PAGE and transferred onto the PVDF membrane (Millipore, USA). The membrane was blocked and incubated with respective primary antibodies (ER and EGFR) at 4 °C overnight. Blots were washed, incubated with HRP-conjugated secondary antibodies, developed using chemiluminescent solution (Immobilon Western, Millipore, USA) and scanned by using gel documentation system (Bio-Rad).

Animals. All procedure involving animal experiments were reviewed and approved (CPCSEA/IAEC/Pharm.Chem.-31/2016-17/129) by the Institutional Animal Ethical Committee (IAEC) of Y. B. Chavan College of Pharmacy, Aurangabad, India. All experiments were performed in accordance with the regulations and guidelines issued by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) India. Virgin female Sprague–Dawley rats (B.W. 150–210 g) were obtained from Wockhardt Research Centre Pvt. Ltd, Aurangabad, Maharashtra, India, and maintained in standard environment conditions (12:12 h light–dark cycle, 25 ± 2 °C, 55 ± 5% relative humidity) with free access to food pellets and water ad libitum. After adaptation for 7 days, rats were randomly divided into 5 groups of six rats each. On the 50th day of rat's age, MNU (50 mg/kg) was injected intraperitoneal (i.p.) (Supplementary file S1). A fresh solution of MNU was prepared by dissolving immediately before use in 0.9% NaCl adjusted to pH 4 with acetic acid.
The normal control group I and MNU cancer-induced group II receive normal saline solution, MNU cancer-induced group III and group IV receive treatment EAFCP at 250 and 500 mg/kg, p.o. body weight, while cancer-induced Group V receive TAM 2 mg/kg, p.o. body weight) for 30 days.

Immunohistochemical analysis. IHC analysis was performed on formalin-fixed, paraffin-embedded tissue sections using standard histologic procedures. The primary antibodies for ER-α was incubated at a dilution of 1:50 for one and half hour at room temperature or 16 h (overnight) at 4 °C. The antigen retrieval was performed with thermic treatment by microwave using 3 cycles of 5 min for ER-α with citrate buffer solution. The tissues were counterstained with hematoxylin. The immunoexpression of ER-α was evaluated as per the Allred score for ER nuclear positivity, the proportion score (PS) (0–5) and the % positive tumor cells are respectively, 0 (0%), 1 (< 1%), 2 (1–10%), 3 (11–33%), 4 (34–66%), and 5 (67–100%). The intensity of staining (IS) for the nuclear positivity of the cells graded as 0, 1, 2, and 3 was as none, mild, moderate, and strong, respectively. So the total scores for ER is given as TS = PS + IS. TS 0 and 2 are negative scores, and 3, 4, 5, 6, 7, and 8 are positive scores.

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Author contributions
All the authors contributed equally. S.N.M. and N.S.S. conducted experiments design and manuscript written. S.A. More conducted network pharmacology analysis. All authors reviewed manuscript for the submission.

Competing interests
The authors declare no competing interests.

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