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# Analysis of human aldehyde dehydrogenases (*ALDH*) gene expression pattern in breast cancer tissue samples: rutin-copper complex inhibit the breast cancer cell proliferation

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## Abstract

**Background:** Higher aldehyde dehydrogenases (*ALDH*) activity is one of the important signatures of breast cancer appearance and has been associated with poor prognosis. *ALDH1A3* has been over-expressed in breast cancer patients. This study aims to analyze gene expression patterns of 18 *ALDH* isozymes in breast cancer tissue samples. It is carried out using a chip-based microarray, next-generation DNA sequencing of *ALDH2* gene following in silico study to identify the natural products which act as inhibitors for over-expressed *ALDH* isoforms. The synthesis of rutin-copper complex and cell viability assay is carried out on MDA-MB-468 cell line.

**Results:** *ALDH1A3* and *ALDH18A1* have shown the highest positive mean fold of variation; whereas, *ALDH2* and *ALDH1A2* have shown the highest negative mean fold variation. In silico studies revealed that rutin has the highest binding affinity with both *ALDH1A3* and *ALDH18A1* and supported with IC<sub>50</sub> value of rutin against MDA-MB-468 cells (144.50 µg/ml). Chemically synthesized rutin-copper complex significantly lowered the IC<sub>50</sub> value to 119.40 µg/ml. The next-generation sequencing study provides the novel single nucleotide polymorphism (SNP) from T to G in the *ALDH2* gene.

**Conclusion:** The present study signifies that, along with *ALDH1A3*, *ALDH18A1* also acts as a marker for breast cancer. Apart from that, inhibitors of *ALDH1A3* and *ALDH18A1* were attained. Perhaps the single nucleotide polymorphism (SNP) obtained during the mutation analysis may be the probable cause of the highest downregulation of *ALDH2* in breast cancer.

**Keywords:** Aldehyde dehydrogenases, Breast cancer, Molecular docking, Rutin

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## Background

Breast cancer (BC) is the second most common cancer that leads to the death of women globally [1]. In India and other Asian countries, the incidence of breast cancer continue to be on the rise and every year nearly half a lakh women lose their lives due to this deadly cancer [2]. The BC tumors, which were not expressing estrogen (ER), human epidermal growth factor receptor (HER2) and progesterone receptor (PR) denote as triple-negative breast cancer (TNBC) [3]. A variety of factors associated with breast cancer like age, family history, lifestyle, hormonal regulation, alcohol consumption, and smoking are chiefly responsible for this pathogenesis [4].

There are several biomarkers used for diagnosis of breast cancer [5]. Over-expressed genes in early stages of breast cancer, such as tumor protein 53 (*TP53*), phosphoinositide-3-kinase-catalytic- $\alpha$  polypeptide (*PIK3CA*), human epidermal growth factor receptor 2 (*HER2*) *ERBB2*, fibroblast growth factor receptor 1 (*FGFR1*), myelocytomatosis oncogene (*MYC*), and phosphatase and tensin homolog (*PTEN*) [6] given in (Table 1) are extensively studied. In humans, aldehyde dehydrogenases (*ALDHs*) are mainly involved in aldehyde detoxification and are also important in the synthesis of retinoic acid [7]. They also play a vital role in alcohol metabolism by removing aldehydes [8]. Among 18 different isoforms of *ALDH*, the activity of each isoform differs from the other according to the localization and tissue distribution.

Several studies have reported that *ALDH* is a specific cancer stem cell (CSC) marker in solid tumors like breast, colon, and lung. The isoforms *ALDH1L1*, *ALDH1A1*, *ALDH1*, *ALDH1A3*, *ALDH2*, *ALDH1A2*, *ALDH3A1*, and *ALDH7A1* have been associated with different types of tumors. Among these genes, *ALDH1A3* which is predominantly expressed in kidney, salivary glands, stomach, and thus over-expressed in breast cancer [9]. Diverse chemotherapeutic treatments also interfere with increased activity of *ALDH*. Certain biomarkers that lead to breast cancer can be used for proper analysis and further treatment [10]. Recent data suggest that there is a high demand for

naturally extracted bioactive compounds for their anticancer properties worldwide [11]. Few findings also suggest that some phytochemicals can stimulate apoptosis in malignant cells in both in vivo and in vitro [12].

The rutin (quercetin glycoside) has tremendous clinical applications which include anti-inflammatory, anti-oxidant, and inhibition of platelet aggregation [13]. Rutin acts as an adjuvant agent that increases the cytotoxic efficiency of two important chemotherapeutic drugs viz., cyclophosphamide (CYC) and methotrexate (MTX) in MDA-MB-231 breast cancer cells [14]. In our present study, we investigated the differential expression patterns of all isoforms of *ALDH* genes in human breast cancer tissue samples and also synthesized the rutin-copper complex to understand the differences in the efficiency of anti-cancer potentials. In silico molecular modelling studies lead to new innovation in designing novel drugs as this study requires less time and can be performed with large quantities of ligands. Besides, it is easy to compare and is found effective in screening [15]. In this study, we have used 12 natural bioactive compounds (Table 2) against *ALDH1A3* and *ALDH18A1* proteins through computational in silico docking. Next-generation sequencing (NGS) was carried out for *ALDH2* gene using illumina platform.

## Methods

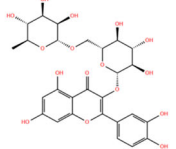
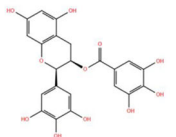
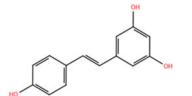
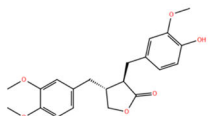
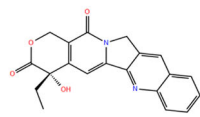
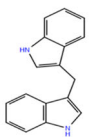
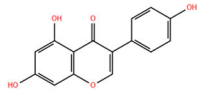
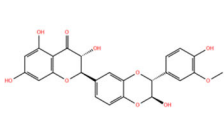
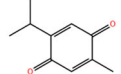
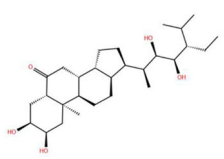
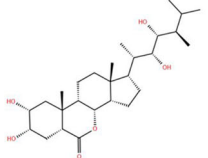
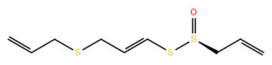
### Selection of patient sample and materials used

RNA isolation kit RNA easy Minikit (Cat-74104), Agilent's Quick-Amp labeling Kit (p/n 5190-0442), TruSeq DNA sample preparation kit (Illumina, #FC-121-1001), Agencourt AMPURE XP beads (Beckman Coulter, #A63881), 7500 Bioanalyzer Chips High Sensitivity Bioanalyzer Chips (Agilent, #5067-4626), Oligo aCGH hybridization kit (Agilent, # 5188-5380), Microarray slide backings (Agilent, # G2534-60005), Human Cot-1 DNA (Invitrogen, #15279-011), Oligo aCGH Wash buffer 1 and 2 set (Agilent, #5188-5226), and Nuclease free water (Ambion, #AM9939),

**Table 1** Overexpressed genes in early breast cancer

Abbreviations	Gene	Function
TP53	Tumor protein 53	Tumor suppressor
PIK3CA	Phosphoinositide-3-kinase-catalytic- $\alpha$ polypeptide	Regulates PI3K signalling. PI3K signalling is important for cell proliferation, migration of cells
ERBB2 (HER2)	Human epidermal growth factor receptor 2 (HER2)	Play an important role in breast cancer progression
FGFR1	Fibroblast growth factor receptor 1	Cell differentiation, growth, proliferation, migration
MYC	Myelocytomatosis oncogene	Acts as protooncogene
PTEN	Phosphatase and tensin homolog	Control cell movement, adhesion of cells to surrounding tissues, and helps in angiogenesis.

**Table 2** Name of natural bioactive compounds and their structures

Name of the compound	Structure
Rutin	
Epigallocatechin	
Resveratrol	
Arctigenin	
Camptothecin	
3,3'-diindolylmethane	
Genistein	
silibinin	
Thymoquinone	
28-homocasterone	
24-Epibrassinolide	
Ajoene	

Bioruptor (Diagenode). Data of the breast cancer patients without any major clinical issues were included and nine tissue samples were collected from Dharwad, India. The tissue samples were stored at  $-70^{\circ}\text{C}$  in RNAlater solution for further experimental use.

#### Isolation of RNA and labeling the target

RNA easy Minikit (Cat-74104) from Qiagen was used to isolate total RNA from tissue samples. RNA integrity analysis, cDNA synthesis, labeling, and microarray methods were carried out sequentially according to Kulkarni et al. [16].

#### Next-generation DNA sequencing

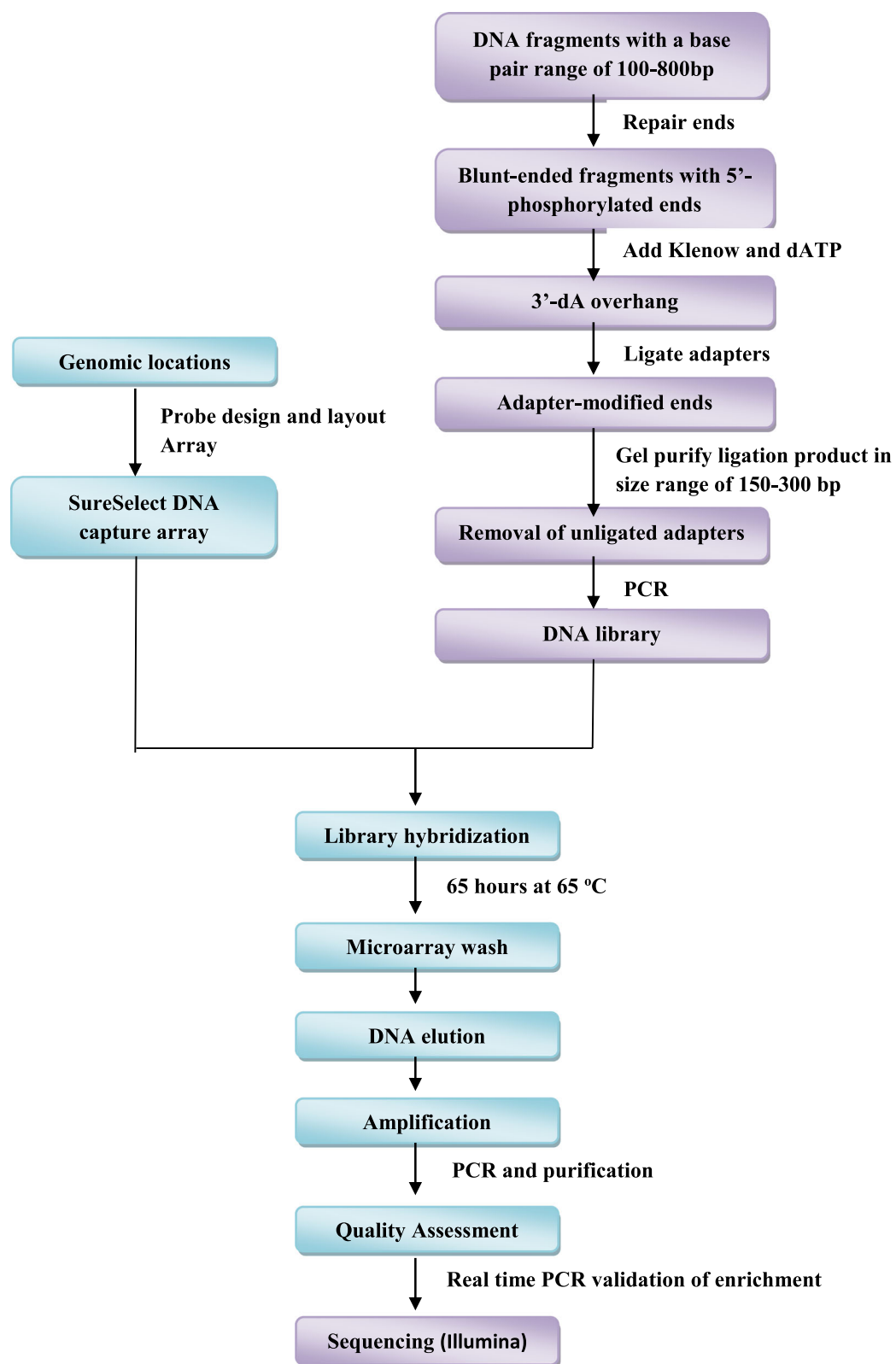
The next-generation DNA sequencing was performed at genotypic technologies private laboratory Bangalore. For this study nine breast cancer tissue samples were collected and used for sequencing of chromosome 12 of gene ID ENSG00000111275 using illumina sequencing platform. 100 bp reads of illumina reads were sequenced using Illumina GAIIX Analyzer. The alignment software Burrows-Wheeler Aligner (BWA version 0.5.9 r16) was used to perform the gapped alignment of illumina sequences against the reference sequence. Alignment software bowtie (version 0.12.7) was used to perform ungapped alignment of illumina sequences against the reference sequence. Samtools (version samtools-0.1.7a) 2 was used for calling SNPs. The procedure flow chart is given in Fig. 1.

#### Synthesis of rutin-copper complex

The stoichiometric mixture of rutin and copper chloride dehydrate of 1mmol and 1.5 mmol were used respectively in methanol solvent and reflux for 5 h. The resulting pale yellow precipitate was dried and washed three times with a mixture of 1:3 (ethanol-water) and was dried. The resultant was used for further experimentation and analysis [17].

#### Cell culture and treatments

Breast cancer cells (MDA-MB-468) were cultured in Dulbecco's modified eagle medium (DMEM) which was supplemented with 10% heat-inactivated fetal bovine serum (FBS) and  $1\times$  anti-myocotic solution. In each well of 96 wells flat bottom plate 200  $\mu\text{l}$  of the cell suspension containing approximately 10,000 cells were seeded and cultured in  $\text{CO}_2$  incubator at  $37^{\circ}\text{C}$  in 95% humidity by supplying 5%  $\text{CO}_2$ . The 100–600  $\mu\text{g/ml}$  concentration of rutin and rutin-copper complex samples was used in the treatment of cells. The  $\text{IC}_{50}$  value for both rutin and rutin-copper complex was calculated after 24 h of experimental incubation time using GraphPad prism version 5.1 [18].

**Fig. 1** Systematic workflow of NGS

**Table 3** Showing the list of fold variation in terms of gene expression profiling in ALDH isoforms

Gene name	Mean fold variation in breast cancer
ALDH3B1	– 0.60
ALDH4A1	– 2.0
ALDH8A1	– 0.18
ALDH6A1	– 2.22
ALDH3A2	– 2.22
ALDH5A1	– 1.26
ALDH9A1	– 1.03
ALDH16A1	– 0.31
ALDH1B1	– 0.52
ALDH1A2	– 3.12
ALDH1L1	– 2.60
<b>ALDH18A1</b>	<b>1.93</b>
<b>ALDH1A3</b>	<b>2.22</b>
ALDH3A1	– 1.03
ALDH3B2	0.42
<b>ALDH2</b>	<b>– 4.19</b>
ALDH7A1	0.24
ALDH1A1	– 2.13

Negative (–) sign indicates down regulation of the gene

### Molecular docking studies

The molecular docking studies were performed on 12 natural compounds against *ALDH1A3* (5FHZ) and *ALDH18A1* (Protein data bank PDBID–2H5G) using Schrödinger Maestro 11.2 version. The 3D structure of the protein *ALDH1A3* (PDB ID–5FHZ) was retrieved from PDB. The protein was optimized accordingly, hydrogen was added and water components surrounding greater than 5 Å were removed. Finally, it was minimized to the default root mean square

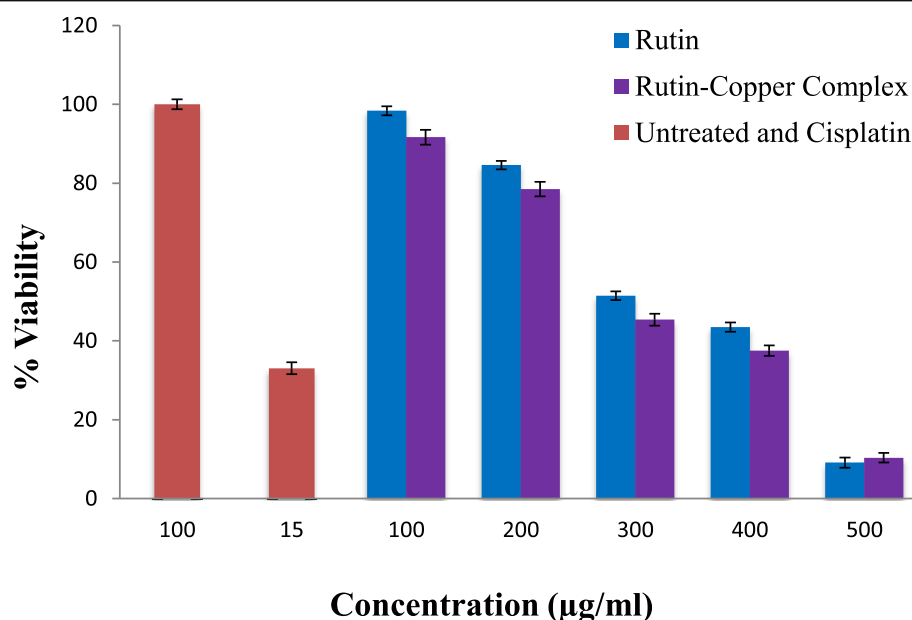
deviation (RMSD) value of 0.30 using protein preparation wizard. Receptor grid generation was performed for minimized protein to identify the binding site of the ligand to the protein. The structures of natural compounds were drawn using 2D sketcher. They were performed using the Ligprep module to generate the 3D tautomer of the compound and to generate all combinations up to 32 per ligand. Ligand docking was performed according to the method of Kalirajan [19]. In Maestro 11.2 version dock, the binding of natural ligands to the active site of the human *ALDH1A3* is an automated process with extra precision (XP) descriptor information and added epik state penalties to docking score. The best bioactive conformation was finalized based on the highest *G* score and highest number of interactions with the protein, mostly the number of hydrogen bonds. The XP visualizer was used as a tool to scrutinize the interactions. The scoring method was the most accepted design, with different parameters like polar, salvation, repulsive, hydrophobic, electrostatic, activity, and *G* score.

### Results

The microarray analysis has given an insight on differential expressions of *ALDH* isoforms in breast cancer tissues. *ALDH1A3* and *ALDH18A1* with highest positive mean fold variation and *ALDH2* with highest negative mean fold variation were noted as depicted in Table 3. These results suggest that *ALDH1A3* and *ALDH18A1* act as biomarkers for breast cancer. Thus, these may be targeted further for designing inhibitors. *ALDH1A3* and *ALDH18A1* with 2.22 and 1.93 fold increases respectively were observed. In contrast, *ALDH2* with – 4.19 fold decrease in expression made us curious to study the DNA sequence of *ALDH2*.

**Table 4** Single-nucleotide polymorphism (SNP) with codon mutation from T to G in *ALDH2* gene in breast cancer samples

Chromosome	Chromosome position	Gene ID	Gene name	Biotype protein coding (PC)	Trancrypt ID	Variation base sample B	SNP quality sample B	Old codon/new codon sample B
chr12	1.12E + 08	ENSG00000111275	ALDH2	PC	ENST00000552234	G	30	gcT/gcG
chr12	1.12E + 08	ENSG00000111275	ALDH2	PC	ENST00000548536	G	30	gcT/gcG
chr12	1.12E + 08	ENSG00000111275	ALDH2	PC	ENST00000549106	G	30	–
chr12	1.12E + 08	ENSG00000111275	ALDH2	PC	ENST00000416293	G	30	gcT/gcG
chr12	1.12E + 08	ENSG00000111275	ALDH2	PC	ENST00000553044	G	30	–
chr12	1.12E + 08	ENSG00000111275	ALDH2	PC	ENST00000546840	G	30	gcT/gcG
chr12	1.12E + 08	ENSG00000111275	ALDH2	PC	ENST00000261733	G	30	gcT/gcG
chr12	1.12E + 08	ENSG00000111275	ALDH2	PC	ENST00000551906	G	30	Tgg/Ggg



**Fig. 2** Effect of rutin and rutin-copper complex on MDA MB-468 cells

### Next-generation sequencing (NGS)

To date, hardly any studies examined the role of *ALDH2\*2* in the development of breast cancer and none of them found any association between *ALDH\*2* in correlation with the risk of breast cancer [20]. These studies have shown G to A point mutation in *ALDH2\*2*. However, in our study, a different novel SNP 'T to G point mutation' was observed in breast cancer samples and is depicted in Table 4.

Effect of rutin and rutin-copper complex on cell viability was analyzed through in vitro studies. The analysis was carried out on MDA-MB-468 cells using cell viability assay 3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT assay) for different time periods. The  $IC_{50}$  value of rutin was 144.50 µg/ml and the rutin-copper complex was 119.40 µg/ml in MDA-MB-468 cells, with a difference of 25.10 µg/ml (Fig. 2). The lesser  $IC_{50}$  value of the rutin-copper complex may suggest a suitable replacement of the rutin to act as an adjuvant.

Molecular docking studies have revealed that rutin had a best binding affinity with both *ALDH1A3* and *ALDH18A1* among different natural bioactive compounds as shown in Figs. 3 and 4. Schrödinger maestro docking scores of ligands against *ALDH1A3* and *ALDH18A1* are given in (Tables 5 and 6), respectively.

### Discussion

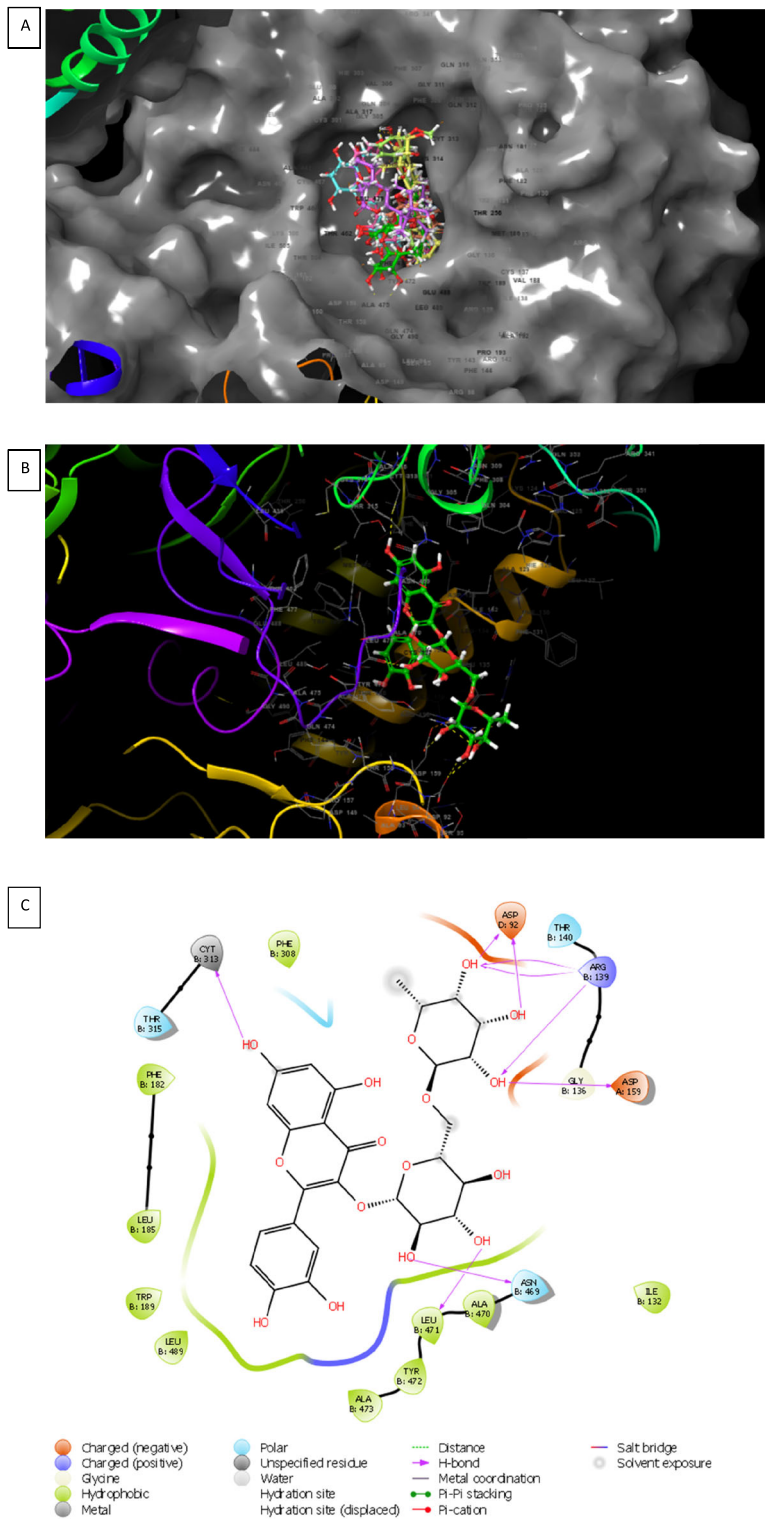
Aldehyde dehydrogenases have a variety of biological effects. It has specifically been linked to different

types of cancer, Parkinson's disease, cataract, obesity, etc. *ALDH1A1* is highly expressed in tissues but not true in case of breast cancer. *ALDH1A3* is a marker for breast cancer patients [21]. In the present study, *ALDH1A3* gene was highly expressed compared to normal as reported earlier [22] but *ALDH18A1* also exhibited high expression in breast cancer. This ultimately proves that *ALDH18A1* also acts as a bio-marker along with *ALDH1A3*. Hence, identifying inhibitors against these may help in better management of breast cancer.

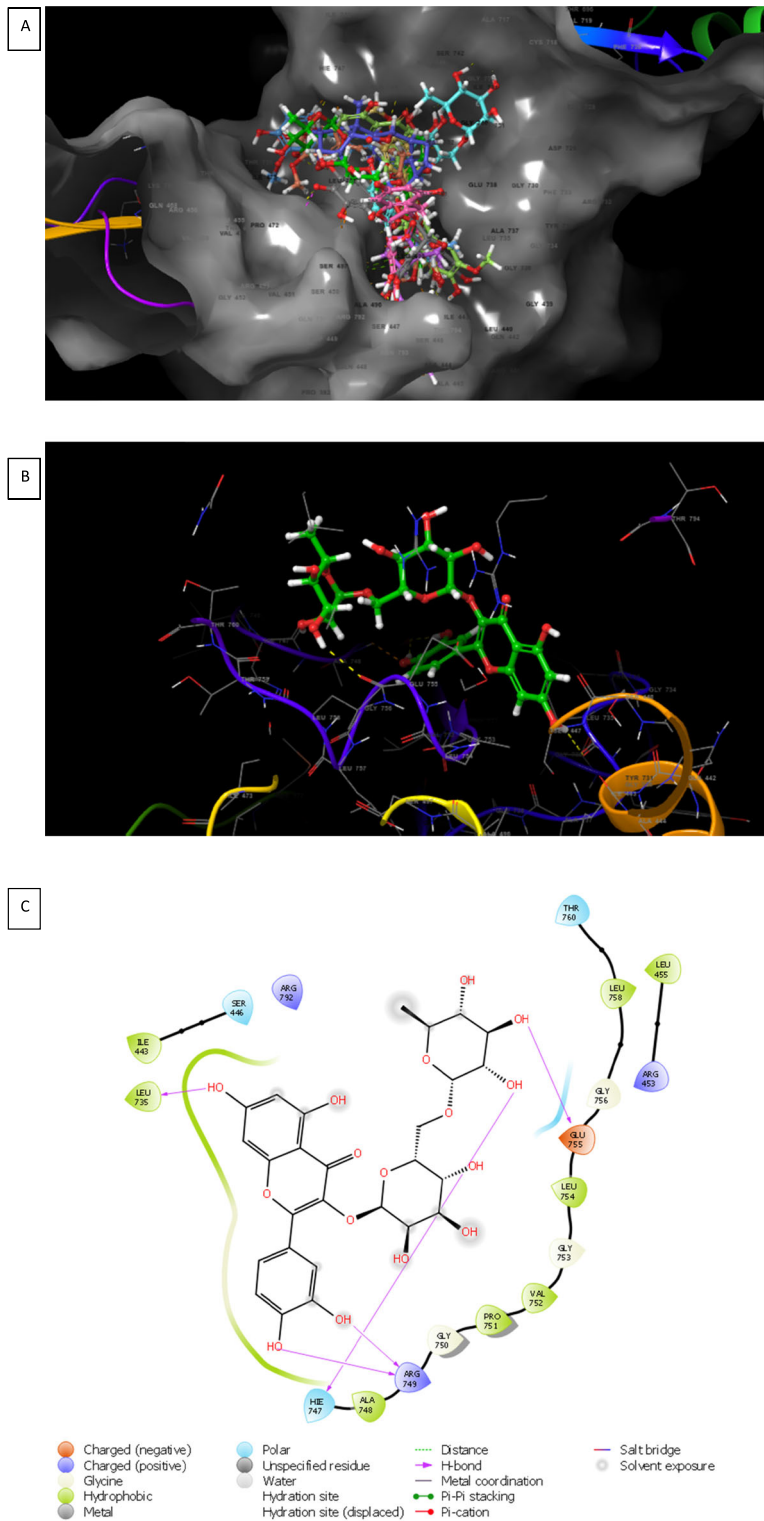
Currently, chemotherapy is widely used to treat a variety of cancers which has its limitations with high toxic side effects to the patients [23]. Hence, research on an alternative approach to treat cancer without side effects is in high demand. Few natural products like flavonoids and polyphenols have better anticancer activity [24]. In silico molecular docking study revealed that rutin has higher binding affinity to both over-expressed *ALDH1A3* and *ALDH18A1* gene among 12 selected natural compounds.

As previously reported, the synergistic action of rutin (glycoside) with other chemo drugs, manifests its potential role as an adjuvant for the treatment of breast cancer patients. Recent study reports on flavonoids complexing with macromolecules to enhance their bio-availability and chemo-preventive efficacy. Rutin (Ru)-fucoidan (Fu) complex was used to overcome the limitations of bio-availability of rutin molecule [25]. Hence, in our current study, a rutin-copper complex was synthesized to study the effects





**Fig. 3** **a** The probable binding mode of all ligands to ALDH1A3. **b** Interaction diagram of rutin ligand with ALDH1A3 protein: the yellow dotted lines in 3D figure indicates the hydrogen bond between the ligand and protein receptor. **c** Two dimensional interaction diagram indicates the rutin interaction with ALDH1A3, one hydrogen bond is between the ligand and amino acid 471 leucine, 469 asparagine, 92 aspartic acid, 159 aspartic acid, and 139 arginine residue in receptor of protein



**Fig. 4** **a** The probable binding mode of all ligands to ALDH18A1. **b** Interaction diagram of rutin ligand with ALDH18A1 protein: the yellow dotted lines in 3D figure indicates the hydrogen bond between the ligand and protein receptor. **c** Two-dimensional interaction diagram indicates the rutin interaction with ALDH18A1, one hydrogen bond is between the ligand and amino acid 735 leucine, two hydrogen bond with 749 asparagine, 755 glutamic acid, and 747 histidine residue in receptor of protein



**Table 5** Schrödinger maestro docking scores of ligands against ALDH1A3

Ligand	G score	Dock score	Lipophilic EvdW	PhobEn	Hydrogen bond	Electro	Site map
<b>Rutin</b>	<b>− 13.68</b>	− 13.67	− 4.28	− 0.4	− 7.17	− 1.93	− 0.05
Epigallocatechin	− 10.41	− 10.35	− 3.96	− 0.2	− 5.32	− 1.07	− 0.32
Silibinin	− 8.94	− 8.94	− 4.49	− 0.19	− 3.53	− 0.84	− 0.17
Genistein	− 8.28	− 8.26	− 4.14	− 0.8	− 2.23	− 0.72	0
24epibrassinolide	− 7.79	− 7.79	− 2.76	− 0.68	− 3.81	− 0.89	0
28homocastasterone	− 7.74	− 7.74	− 5	− 0.44	− 2.21	− 0.21	− 0.14
Arctigenin	− 7.51	− 7.51	− 5.02	− 1.59	− 0.7	− 0.23	0
33'diindolylmethane	− 7.44	− 7.44	− 4.97	− 1.11	− 0.7	− 0.4	0
Resveratrol	− 7.2	− 7.2	− 3.51	− 0.96	− 1.61	− 0.89	0
Camptothecin	− 7.13	− 7.13	− 5.05	− 0.54	− 0.96	− 0.2	− 0.12
Thymoquinone	− 5.1	− 5.1	− 2.56	− 0.64	− 0.7	− 0.1	− 0.6
Ajoene	− 4.09	− 4.09	− 3.4	− 0.98	− 0.7	− 0.27	− 0.38

of both rutin and its complex with copper for their potential bioactivity against breast cancer cells. We noticed that the  $IC_{50}$  value decreased to a large extent for rutin-copper complex compared to rutin. Hence, it suggests that the rutin-copper complex is a lead molecule for further studies in replacing rutin to act as an adjuvant in treating breast cancer patients. Further in silico molecular dynamics simulation study and in vivo studies also act as supportive information to understand the nature of rutin-copper complex.

A rare mutation lys/lys genotype of rare SNP rs671, which is associated with increased breast cancer in Asian women, has been recently reported [26]. However, in this study, the expression of *ALDH2* was very low in breast cancer tissue samples compared to normal breast

tissues. Further, it showed different novel SNP with T to G rather than G to A.

### Conclusion

Like *ALDH1A3*, *ALDH18A1* also acts as a biomarker for breast cancer and a novel *ALDH2* single-nucleotide polymorphism may also be a prognostic indicator for breast cancer in south Indian women. The computational study reveals that Rutin has greater binding effect on both *ALDH1A3* and *ALDH18A1* proteins. The rutin-copper complex has a greater effect on breast cancer cell lines as compared to rutin alone. These results should be further validated with in vivo studies and this study needs further investigation with a large number of patient samples.

**Table 6** Schrödinger maestro docking scores of ligands against ALDH18A1

Ligand	G score	Dock score	Lipophilic EvdW	PhobEn	Hydrngen bond	Electro	Site map
<b>Rutin</b>	<b>− 11.66</b>	− 11.65	− 3.75	0	− 6.38	− 1.58	− 0.11
Epigallocatechin	− 8.37	− 8.31	− 3.42	0	− 4.34	− 1.04	0
Silibinin	− 6.6	− 6.6	− 2.49	0	− 3.28	− 1.15	− 0.09
Resveratrol	− 6.35	− 6.35	− 2.33	0	− 2.8	− 1	0
28homocastasterone	− 5.73	− 5.73	− 2.18	0	− 3.29	− 0.72	0
Genistein	− 5.13	− 5.11	− 2.41	0	− 2.12	− 0.99	0
24epibrassinolide	− 4.97	− 4.97	− 1.61	0	− 2.82	− 1.68	0
Arctigenin	− 4.64	− 4.64	− 2.79	0	− 1.62	− 0.53	0
33'diindolylmethane	− 2.66	− 2.66	− 2.8	0	− 0.7	− 0.15	0
Camptothecin	− 2.35	− 2.35	− 2.11	0	− 0.83	− 0.29	− 0.24
Ajoene	− 2.03	− 2.03	− 2.57	0	− 0.62	− 0.31	− 0.18
Thymoquinone	− 1.39	− 1.39	− 1.2	0	− 0.35	− 0.21	− 0.09

## Abbreviations

ALDH: Aldehyde dehydrogenases; SNP: Single-nucleotide polymorphisms; ER: Estrogen; HER2: Human epidermal growth factor receptor; PR: Progesterone receptor; CSC: Cancer stem cell; CYC: Cyclophosphamide; MTX: Methotrexate; NGS: Next-generation sequencing; FBS: Fetal bovine serum; PDB: Protein data bank; RMSD: Root mean square division; BC: Breast cancer; DMEM: Dulbecco's modified Eagle's medium; BWA: Burrows-wheeler aligner; TNBC: Triple-negative breast cancer

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## Authors' contributions

CMK and PBG designed the research work. SVP, SK, and MRM partake in microarray studies. SVP and MRM partake in in vitro and in silico studies. PHG partake in rutin-copper complex synthesis. BRL and NKP partake in NGS studies. BRL and CMK partake in review and editing. SIM gives directives and technical advices. Finally, all authors have read and approved the research manuscript for publication.

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## Availability of data and materials

Authors SVP and CMK had full access to all the data in the study. Data will be shared to interesting researchers on request.

## Ethics approval and consent to participate

Karnataka Institute for DNA Research (KIDNAR) is the ethical committee which provided us ethics clearance certificate. Ethical approval number: KIDNAR/2015/11/14, B [8].

## Consent for publication

Not applicable

## Competing interests

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

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