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IDENTIFICATION OF POTENTIAL ANTI-TUMORIGENIC TARGETS FOR ROSEMARY COMPONENTS USING DUAL REVERSE SCREENING APPROACHES

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ABSTRACT

Introduction: *Pioneering research on the role of phytochemicals for the treatment of various ailments in the traditional medicines has highlighted rosemary as one of the promising plant for cancer prevention and treatment. The anti-neoplastic properties of rosemary can be attributed to its major bioactive components like Carnosol, Carnosic acid (CA), Ursolic acid (UA), Rosmarinic acid (RA), Rosmanol and other diterpenes. However, only few targets for these bioactive components have identified.* ***Objectives:*** *(1) To identify the molecular targets for carnosol, carnosic acid, rosmarinic acid and ursolic acid.(2)To classify the targets based on their action as anti-inflammatory, anti-apoptotic, signal transduction modulators, antioxidants, antimutagenic, etc. (3) To find the experimental proofs for the each bioactive component and the target.* ***Material and Methods:*** *The putative target identification was done by dual virtual reverse screening approach with the help of two servers namely, PharmMapper and idTatget. The target proteins with anti-neoplastic acivity ranked by fit score and binding energy were obtained and were classified based on their action. The targets obtained were validated from experimental studies.* ***Results:*** *The potential ani-neoplastic targets identified from both the servers were experimentally verified. The study also identified targets for rosemary components which are anti-neoplastic in nature but the experimental proof of their interaction with these components was not studied invivo.* ***Conclusion:*** *The present work using dual inverse screening strategy has revealed anti-tumorigenic targets for 4 bioactive components of rosemary. It also provides tractable set of anti-cancer targets for these components which can be further validated by invivo and invitro study.*

KEY WORDS

Anti-tumorigenic targets, molecular targets, reverse screening, rosemary.

INTRODUCTION

Of the drugs prescribed for cancer treatment majority of them are chemopreventive phytochemicals derived from traditional herbs[1]. The Southeast Asian countries are less prone to different types of cancers due to their dietary habits involving curcumin, onion, garlic, ginger, cruciferous vegetables, tomatoes, chilies etc. that suppress the transformative, hyper proliferative and inflammatory processes that initiate carcinogenesis[2]. The anti-cancer activities of these plants are due to the presence of active components like Curcumin (diferuloylmethane) in Turmeric, Zerumbone in Ginger, isothiocyanates, indole-3-carbinol, isoflavones, protease inhibitors, saponins, phytosterols, inositol hexaphosphate, vitamin C, d-limonene, lutein, folic acid, β-carotene, lycopene, selenium from different types of fruits and vegetables[3].

Rosemary (*Rosmarinus officinalis* L.) a member of Lamiaceae has been studied in the last decade

for many of its therapeutic effects on cenral nervous system (CNS), circulation, hepatoprotective effects, anti-tumorigenic effects etc., its chemo preventive effect is most widely studied[4]. Many researchers have shown its chemopreventive properties on neoplastic cells and experimental evidence indicating towards the anti-carcinogenic and antitumor activities both in *invitro* and *invivo* platforms [5-10, 17-20]. Rosemary's bioactive components include phenolic diterpenes or triterpenes, flavonoids and phenolic acids[8] such as carnosic acid (CA), carnosol, ursolic acid (UA), caffeic acid, betulinic acid, rosmaridiphenol, rosmanol and rosmarinic acid (RA). The antitumor activities of the rosemary constituents are reported for carnosol, CA, UA and RA[9], which have shown to inhibit tumor formation and promotion stages of tumorigenesis[5, 10, 11-13]. The antioxidant and anti-inflammatory properties of the rosemary help the cells of the body to protect from damage. A study conducted on rosemary as a preclinical perspective has revealed that effects of rosemary are not species specific as its effects are consistent both in human cell lines and rodent models[9]. Rosemary has shown to suppress or block pro-inflammatory pathways in different cancer cells[3]. Its extracts have been studied for its antitumor or antineoplastic activities on different types of cancer cell lines/ rat or mice models and only one on human[9]. The probable mechanism for rosemary polyphenols in cancer prevention or progression has been revealed as arresting cell cycle at multiple phases, inhibition of tumor promoting pathways or by inducing cancer preventive markers. Many invivo studies have reported some of the molecular targets for rosemary components for their antioxidant, anti-inflammatory, antiapoptotic targets, signal transduction modulators, antimutagenic and epigenetic activity[3-5, ,9,11-14, 21-22]. Here in this study, we have characterized major pharmacological therapeutic targets to which bioactive components of rosemary bind.

In the present work, we have used comparative study of two reverse screening servers namely PharmMapper and the idTarget to identify the potential anti-tumorigenic targets for rosemary components. Both are open-source online servers which fishes putative therapeutic targets for the given molecule. PharmMapper uses reverse pharmacophore mapping strategy where as idTarget perform divide and conquer approach to predict possible binding targets. Thus dual reverse screening strategy was used to identify the potential anticancerous and anti-tumorigenic targets for the bioactive active components of the rosemary. Reverse screening approach searches a protein database against a given molecule. It involves reverse docking and Pharmacophore mapping which is gaining as quick and computationally rigorous alternative to fish molecular targets.The components of the rosemary bind to many therapeutic targets when searched through Reverse Pharmacology approach using PharmMapper and idTargets. In this study, we have identified and also classified the targets as antimutagenic, anti-inflammatory, antioxidant and signal transducing modulator for four of the rosemary components namely, carnosic acid, rosmarinic acid, carnosol and ursolic acid. The purpose of this work was to identify the potential targets for the bioactive components of rosemary that would help to predict the mechanism of their anti-neoplastic activity *invivo*. Recently several inverse docking tools have been reported such as INVDOCK[15], Tarfisdock[16], PharmaMapper[17], idTarget[18], Inverse Screening @tome server[19]. One such approach was used to identify the potential anti-neoplastic targets of tea polyphenols such as epigallocatechin gallate (EGCG), epigallocatechin

(EGC), epicatechin gallate (ECG) and epicatechin (EC) [20]. In this work we have taken the targets from PharmaMapper and idTargets to analyse the anti-cancerous activity of rosemary bioactive components. The present work has identified anti-tumorigenic targets for 4 bioactive components of rosemary and also provides tractable set of anti-cancer targets for these components which can be further validated by *invivo* and *invitro* study.

MATERIALS AND METHODS

Retrieval of 3D Structures of Rosemary Polyphenols

The three dimensional structures of four components of rosemary namely carnosol, carnosic acid, RA and UA were retrieved from PubChem's database with pubChem IDs CID 442009, CID 65126, CID 5281792 and CID 64945 respectively. The .sdf file formats were then converted to .mol2 using MarvinSketch. This step was essential for both PharmMapper and idTarget servers to predict the molecular targets for active components prior to submission. The.mol2 files were then submitted to both PharmMapper and idTarget for the identification of possible therapeutic targets for each of these components.

Potential Therapeutic Target Identification Using PharmMapper and idTarget

The PharmMapper is a web server (http://59.78.96.61/pharmmapper) that identifies potential target candidates for the given small molecule. During submission a maximum of 300 conformation generations were chosen and only human target set were considered to perform Genetic Algorithm (GA). The pharmocophore targets were then analysed based on the fit score. The idTarget, is also a web server (http://idtarget.rcas.sinica.edu.tw) which predicts possible binding of targets of a small chemical molecule via a divide and conquer docking approach. The idTarget reported an average of 7000 molecular targets for each of these components.

RESULTS AND DISCUSSION

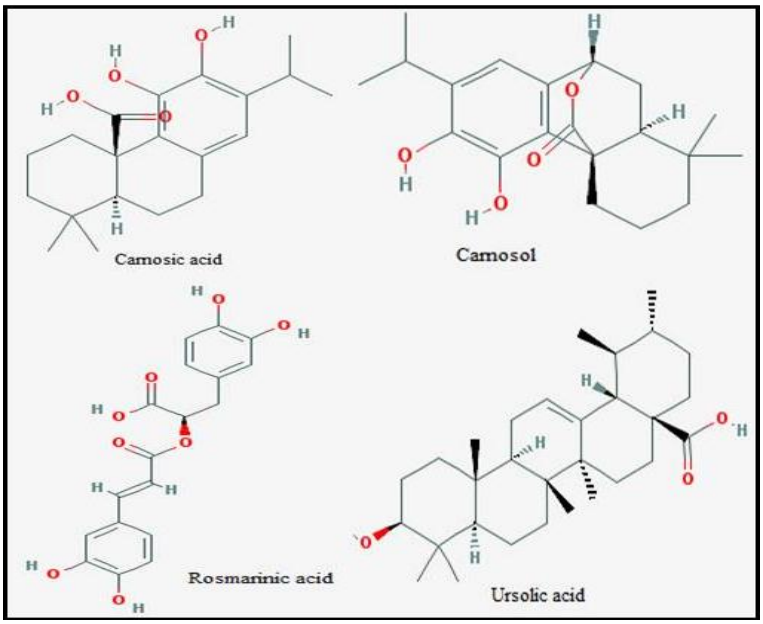

Figure 1. Structure of rosemary components Carnosol, CA, Rosmanol, UA and RA.

The bioactive components studied for anti-cancer targets in this study include carnosol, carnosic acid (CA), ursolic acid (UA) and rosmarinic acid (RA) as shown in **Figure 1**. PharmMapper listed top 300 targtes based on the fit score for each of the component of rosemary. The idTarget server predicted 7527 targets for carnosol, 7535 for CA, 7526 targets for RA and 7278 for UA. Since idTarget server docks given molecule with whole set of PDB database only targets that are predicted by both PharmMapper and idTarget were considered for further analysis.

Potential Anti-tumorigenic Targets for Carnosol

Carnosol is a ortho-diphenolic diterpene which constitutes approximately 5% of the dry weight of the rosemary. Carnosol has been widely studied for its anti-cancer activities [6, 7, 11-14].The potential therapeutic targets for carnosol identified by reverse screening procedures were collected from PharmMapper that gives pharmacologically important targtes. The server predicted majority of targets that are proven experimentally for carnosol. Among the target screened, many were involved in anti-tumor activities which can explain the anti-cancer property of carnosol. The targets so obtained were classified based on their functions as antimutagenic, antioxidant, anti-inflammatory, signal transducing modulators, epigenetic and others. The targets were then confirmed by screening the results of targets predicted by idTarget server. Only those targets which are predicted by both the servers were considered since the approaches used by both the servers are different. There were total 80 targets under these categories predicted by both the servers. 25 targets were of Anti-inflammatory; 41 signal transduction modulators; 02 of anti-mutagenic; 01 epigenetic; 05 anti-oxidant atrgtes and 06 other receptors and enzymes involved in cancer.

Potential targets for carnosol identified by reverse screening procedures and experimentally compared data are listed in **Table 1**. Computed binding free energies and experimental references for several carnosol-protein complexes are also included.

Carnosol binds to proteins of MAPK pathway which are the important targets of inflammation. Of the 80 targets that are predicted by both the servers' majority of them were signal transduction modulators which are involved in inflammation, antioxidant and antiapoptotic activities. Experimentally proven targets for carnosol are listed in the **Table 1**. Carnosol inhibits the early inflammation during cancer progress by binding to Mitogen-activated protein kinase 14 a stress related protein kinase , Cell division protein kinase 2 (CDK2) that controls cell division and Mitogen-activated protein kinase 10 involved in cell proliferation[21,22]. It binds to Glucocorticoid receptor which is the potent anti-inflammatory target[23]. The anti-mutagenic experiment have proved the interaction of carnosol with Glutathione S-transferase A1[24]. A study has shown that carnosol acts as an antagonist by binding to ligand binding site of androgen receptor[25]. Carnosol binds to PPARgamma which inhibits the transcriptional activation of COX-2 a protein produced during transformed cells and human malignancies[26]. This target was predicted by PharmMapper and not by idTarget. Carnosol also binds to Leukocyte elastase , Alcohol dehydrogenase, Glutathione-S-transferase-P, NAD(P)H dehydrogenase [quinone], Catheaspin S , Caspase 3, etc which are also therapeutic targets for cancer are not been been predicted by idTarget. Carnosol interaction with these targets are also not been studied till date. The list of anti-tumor targets to which the carnosol binds is shown along with the

references of targets as anti-tumors in the **Table 2.**

Table 1: Carnosol possible targets found by screening procedures compared with experiment.

Target Name	PDB IDs	Predicted by Server	Implicated by Experiment	Energy Score (kcal/mol)	Reference or Related Disease
Mitogen-activated protein kinase 14	1OUY	PharmMapper/ idTarget	Yes	-8.08	[22]
	2ZB0			-8.9	
	1ZZL			-7.38	
	1BL7			-8.47	
	3FC1			-7.45	
	1ZYJ			-8.11	
	1OZ1			-8.73	
Cell division protein kinase 2 (CDK2)	1PYE	PharmMapper/ idTarget	Yes	-7.97	[22]
	3EZR			-6.92	
	1R78			-7.58	
	2VTP			-7.2	
	2BHE			-8.32	
	1OIQ			-7.95	
Androgen receptor	3B67	PharmMapper/ idTarget	Yes	-9.21	[25]
	1GS4			-9.28	
	1Z95			-9.01	
	2AX8			-8.67	
Glucocorticoid receptor	3CLD	PharmMapper/ idTarget	No	-9.23	Inflammation [23]
	1NHZ			-8.42	
	1M2Z			-9.49	
Mitogen-activated protein kinase 10	1PMN	PharmMapper/ idTarget	Yes	-8.02	[22]
Glutathione S-transferase A1	1GSE	PharmMapper/ idTarget	Yes	-8.06	[24]
	1GUH			-8.45	

Table 2: Other Experimentally proven anti-tumor targets predicted only by ParmMapper to which Carnosol binds

Target Name	PDB IDs	Rank	Reference
PPAR gamma	1RDT 2I4P	146 210	[26]
Glutathione-S- Transferase-P (GST-P)	11GS	83	[24]
Alcohol dehydrogenase class-3	1MA0	95	[27]
NAD(P)H dehydrogenase [quinone]-1	1DXO	142	[28]
Methionine Aminopeptidase	1BOA	8	[29]
Leucocyte elastase	1H18	27	[21]
Cathespin S	1NQC	140	[30]
Mineralocorticoid Receptor	2AA5	177	[31]
Caspase-3	1RHR	184	[32]

HSP90	1UYH	69	[33]
Proteooncogene synthase tyrosine ptotein kinase ABL1	2HZI	263	[34]
Tyrosine kinase ITK/TSK	1SNU	269	[35]
Baculoviral IAP repeat-containing protein 4 (XIAP)	1TFQ	290	[36]
Histone-N-Methyltransferase	1JQE	100	[37]
Vascular endothelial growth factor	1YWN	113	[38]
Proto-ncogene tyrosine protein kinase src	1O4A	221	[39]
Histone deacetylase-8	1T64	261	[40]
3-phosphoinositide-dependent protein kinase 1	2PE1	21	[41]
Cyclin-A2	2IW6	156	[42]

Potential Anti-tumorigenic Targets for CA, RA and UA

The anti-cancer properties of other components of the rosemary have also implicated in number of experiments[3-5,8-10]. Therefore the anti-tumor targets for these components were also studied. The potential anti-tumor targets to which CA, RA and UA are listed In the **Table 3**.

Table 3: Potential anti-tumor targets for Carnosic acid (CA), Rosmarinic acid (RA) and Ursolic acid (UA)

Ligand Name	Target Name	PDB ID	Energy Score kcal/mol
CA	Glucocorticoid receptor	1M2Z	-9.02
	cAMP-specific 3,5-cyclic phosphodiesterase 4B	1XOS	-8.9
	Mitogen-activated protein kinase 14	1OVE	-8.3
	Macrophage metalloelastase	1ROS	-8.74
	Mineralocorticoid receptor	2AAX	-9.24
	Proto-oncogene tyrosine-protein kinase LCK	3BYU	-8.43
	Cell division protein kinase 2	1OIR	-8.43
	Caspase-3	1NMS	-7.83
	cAMP-specific 3,5-cyclic phosphodiesterase 4D	1Y2K	-8.92
	Glutathione S-transferase P	4PGT	-7.79
	Serine/threonine-protein kinase Chk1	2E9U	-7.13
	Vascular endothelial growth factor receptor 2	2P2I	-8.72
	Interleukin-2	1PY2	-8.34
	Estrogen receptor	1YIN	-8.91
	Androgen receptor	1GS4	-8.8
	3-phosphoinositide-dependent protein kinase 1	1UU9	-8.26
	Tyrosine-protein phosphatase non-receptor type 1	1Q1M	-8.15
RA	Cathepsin K	1TU6	-8.47
	Mitogen-activated protein kinase 14	3D83	-8.84
	cAMP-specific 3,5-cyclic phosphodiesterase 4B	1Y2H	-8.33
	Mitogen-activated protein kinase 12	1CM8	-8.99
	Matrix metalloproteinase-9	1GKC	-10.67

	Ras-related protein Rap-2a	1KAO	-10.53
	Caspase-3	1NMS	-9.52
	3-phosphoinositide-dependent protein kinase 1	2PE2	-8.19
	Cell division protein kinase 2	2C6I	-8.27
	Cell division protein kinase 6	1XO2	-9.7
	ADAM 33	1R55	-8.96
	Glutathione S-transferase P	4PGT	-7.42
	Glutathione S-transferase Mu 1	1XW6	-8.15
	Glutathione S-transferase A1	1GUH	-8.1
	Alcohol dehydrogenase class-3	1MC5	-9
	Mast/stem cell growth factor receptor	1PKG	-9.93
	Serine/threonine-protein kinase 6	1MQ4	-9.53
	Trifunctional purine biosynthetic protein adenosine-3	1MEN	-8.86
	Cellular retinoic acid-binding protein 2	1CBS	-8.87
	Serine/threonine-protein kinase Chk1	2HOG	-6.31
	Proto-oncogene tyrosine-protein kinase Src	1O47	-7.61
	Hypoxanthine-guanine phosphoribosyltransferase	1HMP	-7.87
	Tyrosine-protein phosphatase non-receptor type 1	1ECV	-8.85
	Neprilysin	1R1J	-9.73
UA	Glutathione S-transferase P	4PGT	-9.02
	Dipeptidyl peptidase 4	2IIV	-9.12
	Mitogen-activated protein kinase 10	1PMN	-8.95
	Mitogen-activated protein kinase 14	1OVE	-9.32
	Glucocorticoid receptor	3CLD	-9.85
	Macrophage metalloelastase	1ROS	-9.05
	cAMP-specific 3,5-cyclic phosphodiesterase 4B	1XMU	-9.11
	Interleukin-2	1QVN	-8.3
	Cell division protein kinase 2	1OIR	-9.38
	Calmodulin	1CTR	-8.3
	3-phosphoinositide-dependent protein kinase 1	2PE1	-8.91
	Serine/threonine-protein kinase Chk1	2AYP	-7.94
	cAMP-specific 3,5-cyclic phosphodiesterase 4D	1Y2K	-9.23
	Vascular endothelial growth factor receptor 2	1Y6B	-8.32
	Proto-oncogene serine/threonine-protein kinase Pim-1	2BIK	-9.15
	Epidermal growth factor receptor	3BEL	-9.11
	Caspase-3	1NMS	-8.72
	Proto-oncogene tyrosine-protein kinase LCK	1QPE	-8.49
	Glutathione S-transferase A1	1GUH	-8.8
	Estrogen receptor	1XP9	-7.19
	Retinoic acid receptor RXR-alpha	2P1U	-8.24
	E3 ubiquitin-protein ligase Mdm2	1T4E	-9.34
	Tyrosine-protein phosphatase non-receptor type 1	1Q6S	-9.39

The PharmMapper gives the recurring targets with different PDB id. Therefore the above data has been prepared based on the energy score given by idTarget. Only those targets among

them with highest binding energy were considered. The above data are support the anti-cancer properties of the rosemary components. Many of the targets that are listed are have been experimentally proven for the particular rosemary components.

In this study, dual reverse screening approach was used to identify the potential targets for the bioactive components of Rosemary such as carnosol, CA, RA and UA. The results reveal that the reverse screening using PharmMapper and idTarget has characterized those targets for bioactive components of rosemary many of which are experimentally validated as their anti-neoplastic targets. Firstly the result revealed that targets like MAPK-14, CDK2, AR, PPAR gamma, are the experimentally proven for cancer. Screening also identified targets which are clinical targets with anti-cancer effects or enzymes that are involved in antitumor drug design. This work would help to enlighten on the anti-tumorigenic abilities of the bioactive components of the rosemary. The binding potential of rosemary ingredients to their novel set of potential targets can be further validated by *invivo* and *invitro* bioassays. This new reverse screening approach can be used as an alternative computational strategy to for quick identification of potential therapeutic targets in phytochemicals and medicinal plants.

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