RESEARCH ARTICLE

Comparative Reverse Screening Approach to Identify Potential Anti-neoplastic Targets of Saffron Functional Components and Binding Mode

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Abstract

　　Background: In the last two decades, pioneering research on anti-tumour activity of saffron has shed light on the role of crocetin, picrocrocin and safranal, as broad spectrum anti-neoplastic agents. However, the exact mechanisms have yet to be elucidated. Identification and characterization of the targets of bioactive constituents will play an imperative role in demystifying the complex anti-neoplastic machinery. Methods: In the quest of potential target identification, a dual virtual screening approach utilizing two inverse screening systems, one predicated on idTarget and the other on PharmMapper was here employed. A set of target proteins associated with multiple forms of cancer and ranked by Fit Score and Binding energy were obtained from the two independent inverse screening platforms. The validity of the results was checked by meticulously analyzing the post-docking binding pose of the picrocrocin with Hsp90 alpha in AutoDock. Results: The docking pose reveals that electrostatic and hydrogen bonds play the key role in inter-molecular interactions in ligand binding. Picrocrocin binds to the Hsp90 alpha with a definite orientation appropriate for nucleophilic attacks by several electrical residues inside the Hsp90-alpha ATPase catalytic site. Conclusion: This study reveals functional information about the anti-tumor mechanism of saffron bioactive constituents. Also, a tractable set of anti-neoplastic targets for saffron has been generated in this study which can be further authenticated by *in vivo* and *in vitro* experiments.

Keywords: Saffron - crocetin - reverse pharmacophore mapping - reverse docking - target protein - binding mode

Introduction

　　There has been a lot of research interest generated in the last decade on the chemopreventive effect of saffron on neoplastic cells and their upward trends in experimental evidence indicating towards the anti-carcinogenic and anti-tumour activities of saffron and its compounds in *in vitro* and *in vivo* platforms (Morjani et al., 1990; Salomi et al., 1990; 1991; Nair et al., 1991; 1992; 1994; Abdullaev and Frenkel, 1992; Abdullaev, 1994; 2000; Tarantilis et al., 1994; Chang et al., 1996; Escribano et al., 1996; Abdullaev and Gonzalez, 1996; Dufresne et al., 1997; El Daly, 1998; Garcia-Olmo et al., 1999; Escribano et al., 1999a; 1999b; 1999c; 2000; Molnar et al., 2000). A lot of *in vivo* experimental platforms have substantiated the inhibition capability of Saffron extracts in slowing down the growth of tumours (Nair et al., 1991a; 1991b; 1992; 1994; Salomi et al., 1991a; 1991b; El Daly, 1998; Olmo et al., 1999). A research study of skin cancer has shown that topical application of saffron extract has an inhibitory effect on overall progression of dermal carcinogenesis (Salomi et al., 1990; Salomi et al., 1991; Salomi et al., 1991). Also, some research studies have depicted that

there is a significant prolongation in the lifespan of mice treated with cisplatin and there was noticed prevention of decline in body mass, Hb level and WBC Count (Nair et al., 1991). In a pre-clinical cancer chemo-prevention trial where simultaneous administration of cysteine and vitamin E has been carried out, it was noticed that this combination therapy had significant effect in reducing blood urea, nitrogen, and glucose and serum creatinine levels respectively. This therapy also demonstrated a moderate prevention mechanism of cancer induced alterations in the actions of different serum enzymes (El Daly, 1998). Altogether, these studies reflected the potential of saffron as a protective agent in diminishing cisplatin- associated toxic side-effects especially nephro-toxicity. Recently a research study found that crocin; a carotenoid isolated from saffron, improved the survival time and reduced tumour growth in female rats with no considerable effects in male animals (Chang et al., 1996). Therefore, a large number of *in vivo* studies demonstrated that extracts from saffron extract and its purified ingredients considerably augmented the lifespan of rodents with a wide range of tumours. However the precise mechanism of anti-carcinogenic action of saffron has not been pinpointed.

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A wide range of theories has been proposed governing the mode of action of saffron and its bioactive ingredients. The first theory proposed that anti-tumour mechanism of saffron is due to the obstruction of cancer coupled cellular DNA and RNA synthesis, but not protein synthesizing machinery (Abdullaev, 1993; Abdullaev, 2002; Martin et al., 2002). The second theory proposed that saffron's anti-tumour action is because of its inhibitory effect on free radical chain reactions, given that nearly all carotenoids having lipid-soluble property and might have membrane-associated high-efficiency free radical scavenging activity (Nair et al., 1991; 1992; Abdullaev, 1993; Premkumar et al., 2001; Martin et al., 2002; Abdullaev, 2002). The third postulated theory states that the anti-tumour activity is due to the metabolic conversion of naturally occurring carotenoids to retinoids (Takashi et al., 1992; Tarantilis et al., 1994). However, there are recent reports countering this view by stating that conversion of carotenoids to vitamin A is not a necessity for anticancer activity (Smith, 1998). The fourth postulated theory states that the key to the cytotoxic effect of saffron lies with its interaction of carotenoids with Topoisomerase II, an enzyme implicated in cellular DNA-protein interaction (Nair et al., 1991; 1995; Smith, 1998). The fifth proposed theory is associated with the anti-tumour activity of plant-lectins in saffron (Salomi et al., 1991; Oda, and Tatsumi, 1993; Abdullaev and Mejia, 1997; Escribano et al., 2000). Even though numerous theories have been put forward, the precise mechanism(s) of anti-carcinogenic and anti-tumour effects of saffron and its key bioactive ingredients are still unclear.

For most of the phyto-therapeutics the search strategy for molecular targets of bioactive ingredients has been a quite challenging task. The search strategy involving proteomics profiling and pharmacokinetic approaches has been a lengthy and backbreaking procedure (Lin and Lu, 1997; Persidis, 1998; Huang et al., 2004; Hajduk et al., 2005). In this context, a novel reverse pharmacology protocol involving reverse docking and reverse Pharmacophore mapping has found acceptance as speedy and computational intensive alternative method to fish molecular targets. Reverse docking involves inverting the traditional drug discovery pyramid and screening a protein database instead of a drug molecule database. Reverse Pharmacophore mapping is based on the principle of aligning a given small molecule in flexible conformation into each Pharmacophore model of proteins in the target list. In recent times many reverse screening tools have been developed such as INVDOCK (Chen and Ung, 2001), Tarfisdock (Li et al., 2006), PharmMapper (Liu et al., 2010), idTarget (Wang et al., 2012), Inverse Screening@tome server (Pons and Labesse, 2009). Using these computational platforms molecular targets of tea polyphenols viz. epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG) and epicatechin (EC) have been identified (Zheng et al., 2011).

In the present study, we employ a comparative analysis of two different reverse screening approaches, i.e. Reverse Docking system based on idTarget and the Reverse Pharmacophore mapping strategy based on a PharmMapper system, to identify potential molecular targets for saffron bioactive compounds. The putative molecular targets identified by these dual Inverse screening strategies are ranked based on their Fit Score and Binding energies and are further annotated to derive their association to their proposed anti-tumour mechanisms theories. Validation of the docking outcomes is done by further investigation into the binding poses of saffron bioactive compounds and their respective potential targets.

Materials and Methods

Small molecular structures of Saffron bioactive ingredients

The small molecular structures of crocetin, picrocrocin, safranal were retrieved in sdf format from PubChem database. The sdf file format is converted into a mol2 format in MarvinSketch. This step is a prerequisite for submission of the bioactive compounds in PharmMapper and idTarget to conduct the Pharmacophore mapping and Reverse docking procedures respectively.

Reverse pharmacophore mapping

PharmMapper server is an open-source online platform which uses pharmacophore mapping strategy to identify potential molecular target candidates for a given small bioactive molecule. A large, in-house pharmacophore database (namely PharmTargetDB) derived from annotation of all the target information in TargetBank, BindingDB, and DrugBank is hosted by PharmMapper. Once a small molecule is submitted as an input query PharmMapper conduct an automatic search operation to derive the best mapping poses of the query molecule against the entire pharmacophore models in PharmTargetDB. The result of this search operation is a N best-fitted hits library with relevant target annotations along with aligned poses for each target. The mol2 files of crocetin, picrocrocin, safranal were submitted in PharmMapper and a list of possible binding receptors were received. The list was further annotated to screen out the putative target list pertaining to anti-tumour activity.

Reverse docking procedure using idTarget

IdTarget is an open-source reverse docking web platform for predicting possible binding targets of a small chemical molecule using a divide-and-conquer docking approach. This web server requires an input ligand file for the target screening and it possesses two modes for searching binding poses namely: i) Scanning mode, where usual molecular docking procedures are carried out for each protein structure, ii) Fast mode, where the binding sites of the homologous proteins are mapped to ligands by superposition of homologous protein structures with the pre-calculated structural alignment data using CE. The next step is the optimization of binding poses by means of adaptive local searches to eliminate close contacts among protein atoms. The mol2 files of Crocetin, Picrocrocin, and Safranal were submitted in idTarget and a list of possible binding receptors were received. The list was further annotated to screen out the putative target list pertaining to anti-tumour activity of saffron.

Molecular Docking with AutoDock Vina PyRx 0.8

The identified targets that were common in both

Inverse screening tools (PharmMapper and idTarget) were further validated by molecular docking with AutoDock Vina in PyRx 0.8 (Trott and Olson, 2010). AutoDock employs Lamarckian genetic algorithm and a comprehensive scoring function. It takes into account hydrogen bonding and solvation free energy (Morris et al., 1998). AutoDock Vina employs a sophisticated gradient optimization method in its local optimization procedure. Screening of the docking results with AutoDock Vina in PyRx 0.8 was a prerequisite for substantiating the precise molecular mechanism and receptor targeting of saffron bioactive molecules.

Validation of the docking poses in AutoDock

There are numerous success stories demonstrating the accomplishment of AutoDock in docking simulations for reproducing experimental binding structures. In accordance with this principle the structure of Hsp90 α retrieved from RSCS-PDB (PDB ID: 4EGI) and its inbuilt crystallized ligand B2J is re-docked with Hsp90. The molecular interactions of the binding pose of the original B2J-Hsp90 α complex and post-docking simulated B2J-Hsp90 α complex is compared to find out the reliability of AutoDock as an interaction tool in our study. The deviation of bond length distance and RMSD (Root Mean Square Deviation) of Backbone and Heavy Atom between the 'Theoretical docked model' and the experimental derived Crystal complex were also computed.

Results

The therapeutic value of saffron (stigmas of C. *sativus* L.) is established by the presence of three key secondary metabolites: crocin, picrocrocin, and safranal. Saffron is personified for its bitter taste and an iodoform, which are due to the presence of chemicals picrocrocin and safranal. Crocetin is an important carotenoid constituent of saffron and is the major coloring pigment in saffron. In this study we have made an attempt to identify the therapeutic targets of the crocetin, picrocrocin and safranal which gives a combined anti-tumour therapeutic appeal to Saffron. Their chemical structures are displayed in Figure 1.

Potential Protein Targets for Crocetin
Potential protein receptors for Crocetin, Picrocrocin and Safranal identified by dual virtual screening

procedures (Reverse docking via idTarget and Reverse Pharmacology mapping via PharmMapper) compared with experiment data are listed in Table 1. Computed Fit Score and experimental references for several Saffron bioactive molecule-protein complexes are also included. The result shows that this strategy can be successfully implemented in screening out several diseases associated molecular receptors.

Twelve potential receptors whose binding energies rank in the top 3-2% by idTarget and PharmMapper and are associated with cancer inducing pathways are respectively are listed in Table 1. It is of interest that many predicted Crocetin -binding proteins are associated with cancers or tumours. Among them, twelve receptors are screened out by PharmMapper and eight receptors are screened out by idTarget. In these group GTPase HRas, Ras-related protein Rab-5A, Ras-related protein Rap-2a and RAF proto-oncogene serine/threonine-protein kinase targets are related to Ras and Raf signaling pathways. *In vivo* studies have demonstrated that Crocetin has played a therapeutic role in Ras and Raf signaling induced carcinoma in mice (Zhou et al., 2010). Thus our results very well correlate with the experimental evidence. In accordance with the 1st hypothesis that saffron inhibits the cancer associated DNA and RNA synthesis we have identified the molecular targets as DNA mismatch repair protein mutL which is a component of the mismatch repair (MMR) pathway involved in the removal of DNA base mismatches that arise either at some stage in DNA replication or are generated by DNA damage (Martin et al., 2010). Thus we could provide evidence relating to the 1st hypothesis. In accordance with the third hypothesis stating that the trans-retinoic acids in the saffron has an anti-tumour activity we have identified Retinoic acid receptor alpha as a potential receptor. Retinoid-dependent activation of RARα arrests
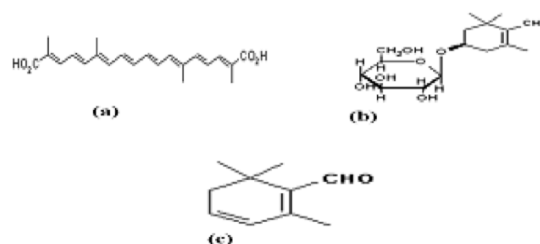

Figure 1. Bioactive Compounds in Saffron. a) Crocetin, b) Picrocrocin, c) Safranal

Table 1. Potential Targets of Crocetin by Screening Procedures Compared with Experiment

PDB_ID	Target Name	Predicted by Procedures	Implicated by Experiment	Fit Score (PharmaMapper)	Binding Energy AutoDock PyRx
1P2S	GTPase HRas	PharmaMapper/idTarget	No	6.897	-4.3
1TU4	Ras-related protein Rab-5A	PharmaMapper	No	5.46	-8.6
1MX0	Type 2 DNA topoisomerase 6 subunit B	PharmaMapper/idTarget	Yes	4.637	Giving error
1B63	DNA mismatch repair protein mutL	PharmaMapper/idTarget	Yes	4.651	-7.3
1KAO	Ras-related protein Rap-2a	PharmaMapper/idTarget	Yes	4.691	2.1
1B55	Tyrosine-protein kinase BTK	PharmaMapper/idTarget	No	4.801	-7.5
1HOV	MMP-2	PharmaMapper/idTarget	Yes	4.605	-5.2
1Z6T	Apoptotic protease-activating factor 1	PharmaMapper	No	4.478	------
1DKF	Retinoic acid receptor alpha	PharmaMapper	No	4.486	0
1C1Y	RAF proto-oncogene serine/threonine-protein kinase	PharmaMapper	No	4.512	------
1PVG	DNA topoisomerase 2	PharmaMapper/idTarget	Yes	4.314	-8.1
1BH5	Lactoylglutathione lyase	PharmaMapper/idTarget	No	4.411	-8.4

the growth, while activation of the other receptor favours the proliferation of the neoplastic cell (Tanaka and De Luca, 2009; Tang et al., 2011). This result also very well correlates with the experimental evidences. The fourth postulated theory states that the key to the cytotoxic effect of saffron lies with its interaction of carotenoids with Topoisomerase II. In our results we also identified Type 2 DNA topoisomerase 6 subunit B and DNA topoisomerase 2 as a potential receptor which very well correlates with the fourth hypothesis. In a post hemorrhage recovery study, administration of crocetin to rodents resulted in increase in the survival period, in addition to having a hepato-protective activity by diminishing the activation of apoptotic cell death (Yang et al., 2011). This study very well correlates with our finding of APAC-F1 (Apoptotic protease-activating factor 1) as a possible binding receptor for inducing apoptosis. The next target identified is MMP-2 and *In vivo* studies have also shown that Crocetin as MMP inhibitors which very well correlates with our finding of its target MMP-2 (Dimitra et al., 2011). The other novel targets identified by the dual Inverse screening protocol but lacking experimental validation were Tyrosine-protein kinase BTK and Lactoylglutathione lyase. In case of chronic lymphocytic leukaemia, an abnormal activation of B-cell receptor (BCR) signaling is observed. Bruton tyrosine kinase (BTK) forms an essential in BCR signaling and BTK inhibitory targeted therapeutics have been shown effective in cancer control (Herman et al., 2011). Glyoxalase I (also known as Lactoylglutathione lyase) is an enzyme that is involved in catalytic isomerization of hemithioacetal adducts, formed spontaneously in reaction with glutathionyl group and aldehydes, for example methylglyoxal (Thornalley, 2003). Several research studies advocates the potentiality of GSH-dependent glyoxalase enzyme system as a key partner in anti-tumour drug development because of that fact that this elementary detoxification pathway induces an increase level of cytotoxic methylglyoxal concentration in tumour cells, and GlxI (glyoxalase I) being the first enzyme in this pathway can be a good targetable proposition (Creighton et al., 2003). These two targets are not found in any experimental validated and needs further *in vitro* and *in vivo* studies to gather concrete evidence about their inhibitory activity.

Potential protein targets for picrocrocin and safranal

Eight potential receptors were identified for Picrocrocin and ten potential receptors were identified for Safranal after the dual Reverse screening procedures by idTarget and PharmMapper as mentioned in Table 2. Many of these identified potential drug targets of safranal and picrocrocin gives an explanation of the mechanism of anti-tumour activity of saffron extracts in prostate and breast cancer induced animal models. We identify that the proposed mechanism of its anti-tumour action in mammary and prostrate tissues is possibly because of binding of safranal and picrocrocin to Estrogen Receptor and Androgen receptors. Another important experimental fact noticed is that saffron inhibits cancer inducing MMP pathways in invitro bioassays. Our dual Inverse screening has revealed that the possible reason for such an activity is due to the

binding of safranal to MMP-13 and thereby acting as an antagonist. There has also been literature published on the anti - tumour activity of saffron on hepatocyte carcinoma in rats (Amin et al., 2011). The possible mechanism of this action identified by our study is the binding of picrocrocin to the Hepatocyte growth factor receptor and thereby arresting the hepatocyte growth factor (HGF)/Met signaling important for pathogenesis of various human cancers.

Other receptors that are identified in our study which have a profound effect on cancer prevention are HSP 90-alpha, Vitamin D3 receptor, HAT ESA1, Galectin-3, Deoxycytidine kinase, p56-LCK, IMPDH-II, MAP kinase 14, PPAR-γ or PPARG and Proto-oncogene serine/threonine-protein kinase Pim-1. Lot of research has been done on the potentiality of inhibitors targeting these receptors to act as cancer chemopreventive agents (Majewski et al., 1996; Reed, 2006; Li et al., 2007; Kock et al., 2007; Hetschko et al., 2008; Floryk and Thompson, 2008; Jones et al., 2009; Yu et al., 2010). However we could not find any published literatures having interaction studies of picrocrocin and safranal on the above mentioned receptors. This opens up new interesting research avenues to test the inhibitory potential of safranal and picrocrocin against these receptors to gather concrete evidence about their anti-metastatic and anti-carcinogenic mechanisms.

Docking results for the original ligand B2J in heat shock protein HSP 90-alpha crystal structure

Hsp90α belongs to Hsp90 family of proteins. They are the key molecular chaperones implicated in signal transduction pathways, cell cycle control, stress management, folding, degradation and transport of proteins. Hsp90α plays the role of a significant mediator in cancer cell invasion and is expressed extracellularly on fibrosarcoma and breast cancer cells and it interacts with MMP2 at that location (Salomi et al., 1991). In many preclinical and clinical research studies a range of Hsp90 inhibitors have demonstrated anti-cancer effect when used as a sole therapeutic agent or when used in combination with chemotherapy. The current generation of

Table 2. Primary and Secondary Targets of Picrocrocin and Safranal

Target Name	Picrocrocin	Safranal
HSP 90-alpha	✓	✓
Vitamin D3 receptor	✓	✓
HAT ESA1	✓	
Galectin-3	✓	
Deoxycytidine kinase	✓	
Proto-oncogene tyrosine-protein kinase LCK	✓	
Hepatocyte growth factor receptor	✓	
Inosine-5-monophosphate dehydrogenase 2	✓	
Androgen receptor		✓
MAP kinase 14		✓
Retinoic acid receptor RXR-alpha		✓
PPAR-γ or PPARG		✓
Retinoic acid receptor beta		✓
MMP -13		✓
Proto-oncogene serine/threonine-protein kinase Pim-1		✓
Estrogen receptor		✓

100.0

6.3

75.0

56.3

50.0

25.0

31.3

Hsp90 inhibitors is grouped into numerous classes based on individual modes of inhibition which are as follows : i) obstructing the binding of ATP at the ATPase catalytic site, ii) interference in cochaperone/Hsp90 interactions, iii) Blocking the receptor interactions of client/Hsp90 alliances and iv) intervention in the routes of PTM in Hsp90 (Neckers, 2006; Li et al., 2009).

Hsp90 alpha contains 232 amino acid residues and has 37% helical (9 helices; 87 residues) and 20% beta sheet (8 strands; 47 residues) arrangement. Although numerous applications have demonstrated the success of AutoDock in docking simulations for reproducing experimental binding structures, it remains a question whether it works for the particular biological system of interest. To examine the reliability of the docking method, the original ligand B2J (4-(ethylsulfanyl)-6-methyl-1,3,5-triazin- 2-amine) from a ligand-Hsp90 alpha complex structure is removed from the PDB file and docks back to the ligand binding site using the same docking strategy as Picrocrocin.

Superimposition of the Post-docked complex structure into the crystallographic structure complex is made to investigate the micro-environment and inter-molecular interactions. In both the structures B2J forms three hydrogen bonds; two bonds with ASP93 and the one bond with SER52 (Figure. 2b). The hydrogen bond lengths of the docked pose is compared with hydrogen bond lengths of crystal structure complex and the deviation of the two was found to be within 0.13 Å. The RMSD (Root-Mean-Square Deviations) of all heavy atoms of B2J between the docked poses and the crystallographic coordinates range from 0.3-1.6 Å. These results show that the docking method is able to reproduce the experimental binding structure of ligand-Hsp90 alpha complex.
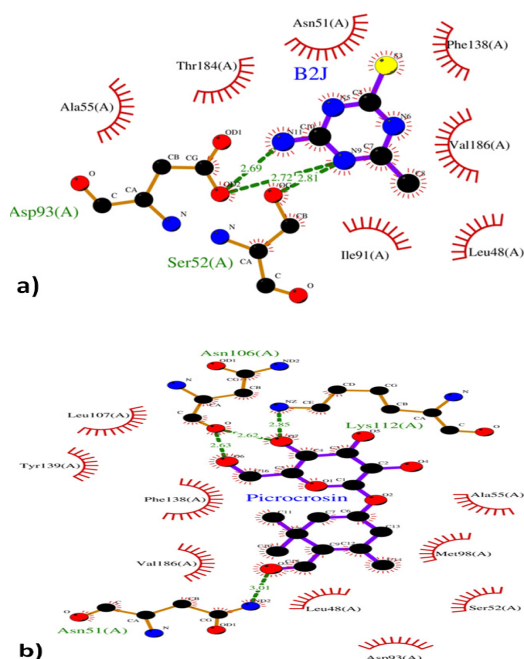

Figure 2. Micro Environment of the Bind Site of B2J/ Picrocrocin and Hsp90 Alpha. a) The original ligand B2J forms 3 hydrogen bonds with residues ASP93, SER52 (green colour dotted lines), b) Picrocrocin binds to Hsp90 alpha in the active cavity (catalytic center and binding domain) where the original ligand B2J located and forms 4 hydrogen bonds with ASN51, ASN106, and LYS112

Binding Mode between Hsp90 and Picrocrocin

The post-docking binding conformation of B2J and picrocrocin with Hsp90 alpha has been illustrated in Figure 2, with the binding free energy -4.83 and -5.89 kcal/mol respectively. In Figure 2a we can notice that, picrocrocin binds to Hsp90 alpha in the active cavity where the original substrate B2J located. In this figure we can also notice that two benzene ring planes of Picrocrocin are located in the active cavity composed of electrical amino acids such as Asp93, Ser52, Leu48, Met98, Ala55, Val186, Phe138, Tyr139, Leu107, etc. represented in brown colour. Alternatively, polar residues ASN51 and LYS112 , coloured in yellow, form 2 hydrogen bonds with N…O—H and polar residue Asn106 forms two hydrogen bonds with O…O—H having bond distance 3.01 Å,2.85 Å and 2.62 Å, 2.63 Å respectively. Investigation of the binding mode evidently exhibits an interactions pattern mediated by electrostatic and hydrogen bonds play a key role in the binding of picrocrocin and Hsp90 alpha and suggests that picrocrocin act as a competitive inhibitor in the ATPase site of Hsp90 alpha.

Discussion

In conclusion, in this study, a comparative Inverse screening approach using idTarget and PharmMapper is done to identify the potential receptors for Saffron bioactive substances such as Croctein, Picrocrocinand Safranal. Our results reveal a number of interesting facts. Firstly a number of receptors of saffron bioactive constituents identified by our method such as MMP-3, MMP-13, and Topoisomerase II have been well established in experiments setting. Secondly other receptors identified by us also fall under conventional clinical targets with anti-tumour effects or target enzymes of drug design. To validate the scheme of identification we further explore the binding mode between an identified receptor Hsp90 alpha and picrocrocin and its co-crystallized ligand B2J. The binding pose revealed that electrostatic interaction and hydrogen bonds facilitate the binding of picrocrocin in the ATPase catalytic site of Hsp90 alpha and can possibly act as a competitive inhibitor and a potential anti-tumour drug. Further *in vivo* and *in vitro* bioassays testing have to be done to gather concrete evidence about the binding potential of saffron ingredients to a novel set of characterized targets. This study provides an alternative computational methodology for rapid identification of therapeutic targets in phytochemicals and medicinal plants.

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