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## Research Article

# Anti-angiogenic Activity of Major Phenolics in Tamarind Assessed with Molecular Docking Study on VEGF Kinase Proteins

<sup>1</sup>Alaadin El-Sayed El-Haddad, <sup>1</sup>Amr Mohammed Saadeldeen and <sup>2</sup>Soad Zakaria El-Emam

<sup>1</sup>Department of Pharmacognosy, Faculty of Pharmacy, October 6 University, Giza, Egypt

<sup>2</sup>Department of Pharmacology and Toxicology, Faculty of Pharmacy, October 6 University, Giza, Egypt

## Abstract

**Background and Objective:** The waste products of the tamarind canning industry have been discarded; however, it has potential health benefits. Herein, the study was carried out HPLC profiling of phenolic constituents of *Tamarindis indica* pericarp and seeds. Furthermore, the cytotoxic activity against HUH-7 cells was evaluated and assessed with molecular docking study on angiogenesis-related VEGF kinase proteins in addition to evaluating the level of released VEGF in treated HUH-7 cells by ELISA. **Materials and Methods:** Folin-ciocalteu and  $\text{AlCl}_3$  assays were used for quantification of total phenolics (TPC) and total flavonoids (TFC) contents, respectively. Molecular docking study was done on VEGF kinase proteins. **Results:** TPC and TFC of pericarp and seeds were  $0.35 \pm 0.02 \text{ g GAE g}^{-1} \text{ DE}$  and  $0.12 \pm 0.009 \text{ g CE g}^{-1} \text{ DE}$ ,  $0.39 \pm 0.01 \text{ g GAE g}^{-1} \text{ DE}$  and  $0.03 \pm 0.006 \text{ g CE g}^{-1} \text{ DE}$ , respectively. In pericarp, 8 phenolics were tentatively identified, where (+)-catechin was the major ( $27,386.04 \mu\text{g g}^{-1} \text{ DE}$ ) followed by gallic acid and naringenin ( $931.47, 500.42 \mu\text{g g}^{-1} \text{ DE}$ ) respectively. While in seeds, 11 phenolics were tentatively identified, where naringenin was the major ( $95,305.47 \mu\text{g g}^{-1} \text{ DE}$ ) followed by (+)-catechin and rutin ( $54,930.29, 15,361.66 \mu\text{g g}^{-1} \text{ DE}$ ) respectively. Aqueous and methanol seeds extracts exhibit cytotoxic effect with  $\text{IC}_{50} 27.4 \pm 1.81$  and  $13.4 \pm 0.94 \mu\text{g mL}^{-1}$ , respectively, it was more potent than aqueous and methanol pericarp extracts which had  $\text{IC}_{50} 132 \pm 5.82$  and  $61.6 \pm 3.16 \mu\text{g mL}^{-1}$ . The tested phenolics were fit on the active sites of VEGF kinase targets with varied degree of interactions. The cytotoxic and anti-angiogenic activities were confirmed in light of phenolics docking interactions. **Conclusion:** Results demonstrate for the first time that phenolics could inhibit angiogenesis via inhibiting kinase proteins, which could therefore be developed as antiangiogenic drugs.

**Key words:** Cytotoxicity, HUH-7 cells, phenolics, *Tamarindis indica*, VEGF

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**Corresponding Author:** Alaadin El-Sayed El-Haddad, Department of Pharmacognosy, Faculty of Pharmacy, October 6 University, Giza, Egypt

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**Competing Interest:** The authors have declared that no competing interest exists.

**Data Availability:** All relevant data are within the paper and its supporting information files.

## INTRODUCTION

The anticancer agents should target multiple pathways that involved in carcinogenesis. Angiogenesis is a necessary process in tumor growth and metastasis. The angiogenic factor, vascular endothelial growth factor (VEGF), is a cell signaling protein that initiates vasculogenesis (formation of new blood vessels) and its binding to the receptors (VEGFRs) stimulates endothelial cell survival, proliferation and migration<sup>1</sup>. A new tactic of anticancer drugs by inhibition of angiogenesis have been identified. VEGF signaling pathway has been considered as a promising target in controlling cancer progression<sup>2</sup>. Recently, inhibitors against VEGF pathway (e.g., sunitinib) that are released in the market elicited a range of adverse effects<sup>3</sup>. Thus, devising safe new anti-angiogenic drugs with selected anti-tumor targets is necessary. Dietary phytochemicals as chemopreventive agents are expected to be less toxic. Natural polyphenolics; catechin, naringenin and procyanidin are well-known to exhibit beneficial health effects including anti-inflammatory, anti-proliferative and antitumor activities. Procyanidin has numerous targets of intracellular signaling pathways, including pro-inflammatory mediators of cell survival, apoptosis and angiogenesis<sup>4</sup>. Currently, many reports suggest procyanidin as a promising compound for cancer prevention and treatment.

Tamarind (*Tamarindis indica* L., Caesalpinioideae) is widely consumed fresh or as juice and jams. Antioxidant, antihyperlipidemic<sup>5</sup> and antimicrobial<sup>6</sup> activities were reported for its fruits. The fruit extract was more active than that of the seeds, with greater ability to scavenge radicals and inhibit xanthine oxidase<sup>7</sup>. Phenolics and flavonoids viz; catechin, epicatechin and taxifolin were isolated from the fruits extract<sup>7</sup>. Tamarind seeds were reported to contain mainly procyanidin oligomers (up to eleven)<sup>7</sup>. Flavonoids can reduce lipid oxidation by lipoxygenase inhibition or by radicals scavenging<sup>8</sup>. Naringenin has been demonstrated to exhibit antioxidant and antiproliferative effects<sup>9</sup>.

A large amount of tamarind seeds wastes have been discarded from the industry. Tamarind seeds are a low-cost rich source of antioxidants phytochemicals such as catechin and naringenin<sup>10,11</sup>. The study's goal was to highlight the potential health benefits of underutilized by-product. Herein, quantification and HPLC profiling of phenolic constituents of *T. indica* pericarp and seed extracts have proceed. Furthermore, in continued search for cytotoxic phytochemicals<sup>12</sup>, the cytotoxic activity was evaluated and assessed by molecular docking of major occurring phenolics in tamarind on VEGF kinase proteins.

## MATERIALS AND METHODS

The study was proceed from May, 2017 till June, 2019 in Faculty of Pharmacy, October 6 University and HPLC profiling was done on National Research Centre, Cairo, Egypt. Gallic acid, (+)-catechin, Folin-Ciocalteu's reagent, MTT (5 mg mL<sup>-1</sup>) in 0.9% NaCl solubilized in acidified isopropanol (0.04 N HCl in isopropanol) (Sigma-Aldrich Co., Germany). Authentic phenolics for HPLC profiling were kindly supplied by Food Technology Research Institute, Giza, Egypt. All other chemicals were of the highest purity.

**Plant material and extraction process:** Pods of *T. indica* L. were purchased from Egypt's local market. The identity was kindly confirmed by Agriculture Research Center, Cairo, Egypt. Seeds were carefully separated from pericarp and were powdered separately. Powdered pericarp and seeds (100 g each) were separately defatted with n-hexane (3 × 1 L) then extracted in Soxhlet with methanol (99.5%, 500 mL, 30 min). In a parallel manner, another powdered pericarp and seeds (100 g each) were separately defatted and extracted in Soxhlet with water (500 mL, 30 min). Extracts (methanol and aqueous) were left to cool, filtered then were evaporated individually (Rotavapor® R-300, BÜCHI, Switzerland)<sup>13</sup>.

**Total phenolics and flavonoids contents:** Total phenolics content (TPC) as gallic acid equivalent of dried extract (GAE g<sup>-1</sup> DE) and total flavonoids content (TFC) as catechin equivalent (CE g<sup>-1</sup> DE) were carried out using folin-ciocalteu and AlCl<sub>3</sub> assays, respectively. Dried powdered *T. indica* pericarp and seeds (1 g each) were separately defatted with n-hexane (10 mL, twice) followed by extraction by sonication with methanol (95%, 50 mL). Methanol was distilled and extracts volume were adjusted to 100 mL with distilled water for phenolics determination or with ethanol for flavonoids determination. By measuring the color intensity using spectrophotometer (Shimadzu Corporation, Japan), phenolics and flavonoids were complexed with folin-ciocalteu's phenol and AlCl<sub>3</sub>, respectively, with reference to pre-established standard calibration curves<sup>14,15</sup>.

**HPLC profiles of phenolics:** Methanol extracts (1 g each) were separately sonicated with aqueous acetone (70%, 200 mL). After acetone removing, the residues were sonicated in methanol (10 mL, 5 min) then centrifuged (1000 rpm, 10 min). The supernatants were filtered (0.2 millipore membrane filter). Identification was performed using Hewlett Packard HPLC system (series 1050) equipped with a Lichrosorb RP-18 column

(4.0×250 mm; 5 µm) and UV detector (280 and 330 nm for phenolics and flavonoids, respectively). Elution was carried out using methanol and acetonitrile (2:1, 1 mL min<sup>-1</sup>). Peaks assignments were confirmed by the injection of authentic. The retention time and peak area were used to calculate compound concentrations by Hewlett Packard software<sup>16,17</sup>.

### **In vitro cytotoxic activity and evaluating the level of VEGF**

**release:** Cytotoxic activity was valued by MTT cell proliferation assay<sup>18</sup>. In 96 well-microplates, human liver carcinoma (HUH-7) cells (0.5×10<sup>5</sup>) were cultured. Cells were treated with tamarind extracts individually dissolved in DMSO (20 µL, 100-0.8 µg mL<sup>-1</sup>). After incubation of plates, the media were removed and MTT solution (40 µL/well) was added and the plates were shaken at room temperature (4 h). Absorbance was photometrically determined at 570 nm using ELISA reader (FLUOstar Omega, BMG, Labtech, Germany). The relative viability percentage was calculated by<sup>18</sup>:

$$\text{Relative viability (\%)} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \times 100$$

For VEGF quantification, HUH-7 cells were cultured in RPMI media, treated with tamarind extracts (100 µg mL<sup>-1</sup>) and incubated (12 h). Briefly, after incubation, media and cells were collected and centrifuged. The cell lysate was prepared and passed through a 21-gauge needle to break up cell aggregates, then centrifuged (14,000 rpm, 15 min, 4°C). The supernatants were used for quantifying VEGF by Sandwich ELISA using a VEGF ELISA kit according to the manufacturer's instructions (Cusabio Technology LLC)<sup>19</sup>. Data (as Mean±SD) were statistically analyzed by GraphPad Prism 6 (La Jolla, CA, USA), using one-way ANOVA followed by Tukey's Kramer Multiple Comparison Test.

### **Molecular docking study**

#### **Preparation of targets and molecular docking protocols:**

Molecular Operating Environment (MOE 2014.0901) software was used. The X-ray crystallographic structure of VEGF-1 (PDB ID: 3HNG), VEGF-2 (PDB ID: 2QU6, 2QU5, 2P2I, 3EWH, 1YWN) transferase (kinase) proteins were downloaded (<https://www.rcsb.org>). The enzymes were prepared by removal of unnecessary chains and/or ligands. Docking setup was validated by redocking of the co-crystallized ligands for predicting the ligand-receptor interactions. The enzymes and examined compounds were then 3D protonated and energy minimized with default options. The protocol suitability was assured by low binding energy score (S), small RMSD value

Table 1: Redocked conformations of co-crystallized ligands with VEGF kinase proteins

VEGF proteins	ID of ligands	RMSD values (Å)
3HNG	85T	0.301
2QU6	857	0.90
2QU5	276	0.40
1YWN	LIF	0.56
2P2I	608	0.32
3EWH	K11	0.30

and the ability of the redocked pose to reproduce the key interactions accomplished by the co-crystallized ligand, (Table 1). The validated protocol was then used in predicting the ligand-receptor interactions at the binding site for catechin, naringenin and procyanidin B2.

## **RESULTS**

### **Phenolics and flavonoids contents and HPLC profiling:**

In pericarp, TPC and TFC were 0.35±0.02 g GAE g<sup>-1</sup> DE and 0.12±0.009 g CE g<sup>-1</sup> DE, respectively. However those for seeds were 0.39±0.01 g GAE g<sup>-1</sup> DE and 0.03±0.006 g CE g<sup>-1</sup> DE, respectively (Table 2). This is similar to the levels reported previously<sup>8</sup>. The TPC differed between pericarp and seeds extracts as always TFC is lower than TPC<sup>20</sup>.

The methanol extracts obtained from the tamarind pericarp and seeds were analyzed by HPLC-UV. Relative concentrations were determined by peak areas (Fig. 1). In pericarp, eight phenolics were tentatively identified, where (+)-catechin was the major (27,386.04 µg g<sup>-1</sup> DE) followed by gallic acid and naringenin (931.47, 500.42 µg g<sup>-1</sup> DE) respectively, in addition to syringic acid, propyl gallate, daidzein (4',7-dihydroxyisoflavone), quercetin and cinnamic acid (Table 3, Fig. 1a). While in seeds, eleven phenolics were tentatively identified, where naringenin was the major (95,305.47 µg g<sup>-1</sup> DE) followed by (+)-catechin and rutin (54,930.29, 15,361.66 µg g<sup>-1</sup> DE) respectively, in addition to gallic, caffeic, syringic, coumaric acids, vanillin, propyl gallate, quercetin and cinnamic acid (Table 3, Fig. 1b).

### **In vitro cytotoxic activity and evaluation of VEGF released**

**by HUH-7 cells:** Tamarind pericarp and seeds extracts were examined against HUH-7 cells. Aqueous and methanol seeds extracts exhibited cytotoxic effects with IC<sub>50</sub> 27.4±1.81 and 13.4±0.94 µg mL<sup>-1</sup>, respectively, it was more potent than those of pericarp extracts (IC<sub>50</sub> of 132±5.82 and 61.6±3.16 µg mL<sup>-1</sup>, respectively) (Table 4). Treatment of HUH-7 cells with tamarind extracts significantly (p<0.05) reduced the level of VEGF when compared with untreated control cells. Moreover, further inhibition was observed with

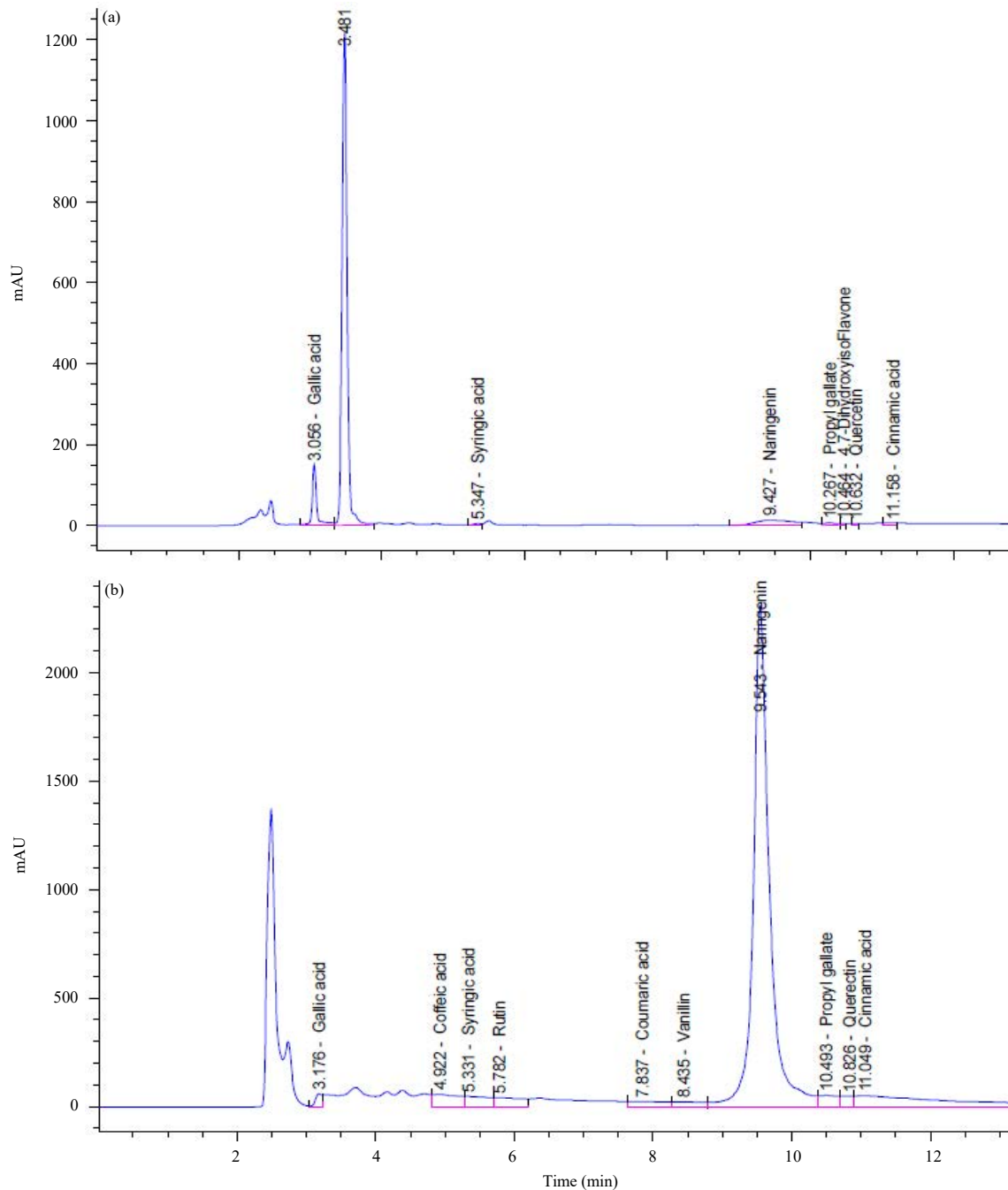


Fig. 1(a-b): Analytical HPLC-UV chromatograms of (a) Tamarind pericarp and (b) Seeds methanol extracts

seed extracts especially the methanol extract when compared with pericarp extracts (Fig. 2). Our results are in accordance with several studies showing the significance of phenolics in inhibiting angiogenesis via reducing VEGFR signaling<sup>4,21</sup>.

**Molecular docking of detected phenolics on VEGF kinase proteins:** Molecular docking of catechin, naringenin and

procyanidin B2 were performed for the first time on VEGF kinase enzyme. Tested phenolics were fitted on the active sites with varied degree of interactions. The active pocket of 3EWH is mainly composed of conserved ASP 1046, GLU 917, PHE 1047 and LYS 868. The main interactions include H-bonds and H-pi interactions between amino acids and ligand. Catechin interacts by four H-bonds between three hydroxyl groups and

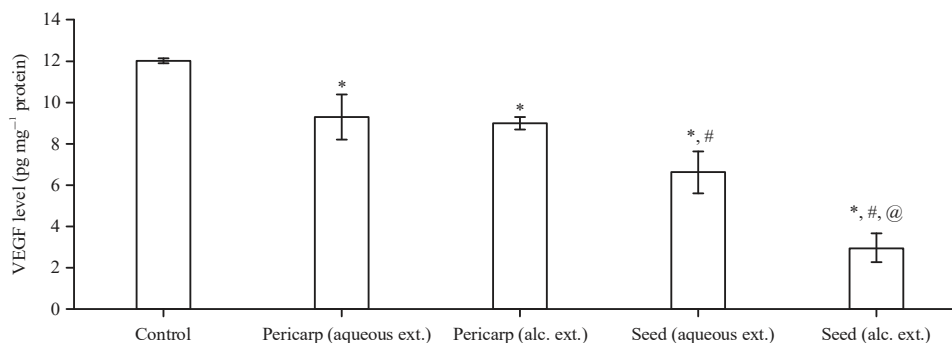


Fig. 2: Level of VEGF released by HUH-7 cells after treatment with tamarind pericarp and seeds extracts

\*p<0.05 when compared to untreated control cells, #p<0.05 when compared to pericarp extracts treated cells, @p<0.05 when compared to seed aqueous extract treated cells

Table 2: Total phenolics (TPC) and total flavonoids (TFC) contents of tamarind pericarp and seeds

Extracts	Contents (µg g <sup>-1</sup> DE)	
	Total phenolics contents (TPC)	Total flavonoids contents (TFC)
Pericarp	0.35 ± 0.02	0.12 ± 0.009
Seeds	0.39 ± 0.01	0.03 ± 0.006

Table 3: Phenolics contents of tamarind pericarp and seeds analyzed by HPLC-UV

Compounds	Contents (µg g <sup>-1</sup> DE)	
	Pericarp	Seeds
Gallic acid	931.47	2,149.18
(+)-catechin	27,386.04	54,930.29
Coffeic acid	nd	4,352.65
Syringic acid	20.52	3,852.8
Rutin	nd	15,361.66
Coumaric acid	nd	2,087.01
Vanillin	nd	2,116.41
Naringenin	500.42	95,305.47
Propyl gallate	49.55	2,149.92
Daidzein	11.53	nd
Quercetin	50.55	3,944.42
Cinnamic acid	12.93	5,129.57

nd: not detected

Table 4: IC<sub>50</sub> of *T. indica* pericarp and seed extracts against HUH-7 cells

Extracts/standard	IC <sub>50</sub> (µg mL <sup>-1</sup> )	
	Aqueous extract	Methanol extract
Pericarp	132.0 ± 5.82	61.6 ± 3.16
Seeds	27.4 ± 1.81	13.4 ± 0.94
Doxorubicin		16.1 ± 0.82

ASP 1046, GLU 917 and PHE 1047 moreover it also interacts by pi-H-bond with LYS 868 (Table 5, Fig. 3). However, in 3HNG, the active pocket is mainly composed of conserved GLU 878, ASP 1040 and LYS 861. Its main key interactions include H-bonds and pi-H-bond interactions. Naringenin interacts by two H-bonds, one is between the hydroxyl group and GLU 878 and the other is between the carbonyl group and ASP 1040,

moreover it also interacts by pi-H-bond with LYS 861 (Table 5, Fig. 4). The binding pocket of 2QU6 is mainly composed of conserved GLU 885, GLY 1048, HOH 1 and HOH 200. The main key interactions include H-bonds and pi-H-bond interactions. Procyanidin B2 interacts by two H-bonds between hydroxyl groups and GLU 885 and GLY 1048. It also interacts by pi-H-bond with GLU 885, moreover it interacts through two hydroxyl groups with water-mediated H-bond with HIS 1026 and ILE 1025 (Table 5, Fig. 5). The cytotoxic and anti-angiogenic activities of tamarind extracts were confirmed in light of its phenolics docking interactions.

## DISCUSSION

Phenolics and flavonoids are naturally-occurring phytochemicals, exhibited good antioxidant potential. To date, more than 6000 flavonoids have been recognized. Methanol, as a polar solvent, efficiently used in the extraction of phenolics<sup>22</sup>. RP-HPLC-UV analysis of the extracts revealed the presence of several peaks corresponding to phenolics and flavonoids including naringenin, (+)-catechin, rutin, gallic, coffeic, syringic, coumaric acids, vanillin, propyl gallate, quercetin and cinnamic acid (Table 3). Catechin and naringenin were the majors in pericarp and seeds extracts respectively. Sudjaroen *et al.*<sup>7</sup> reported that the pericarp mainly contains catechin and naringenin, while the seeds are mainly dominated by proanthocyanidins and naringenin and lower levels of catechin.

All of the detected phenolics are known to have biological activities viz; (+)-catechin, has chemoprotective effects by reducing oxidative stress. The detected flavonoids could act synergistically in antioxidant activities. Result of *in vitro* anti-carcinogenesis assay showed that naringenin inhibited

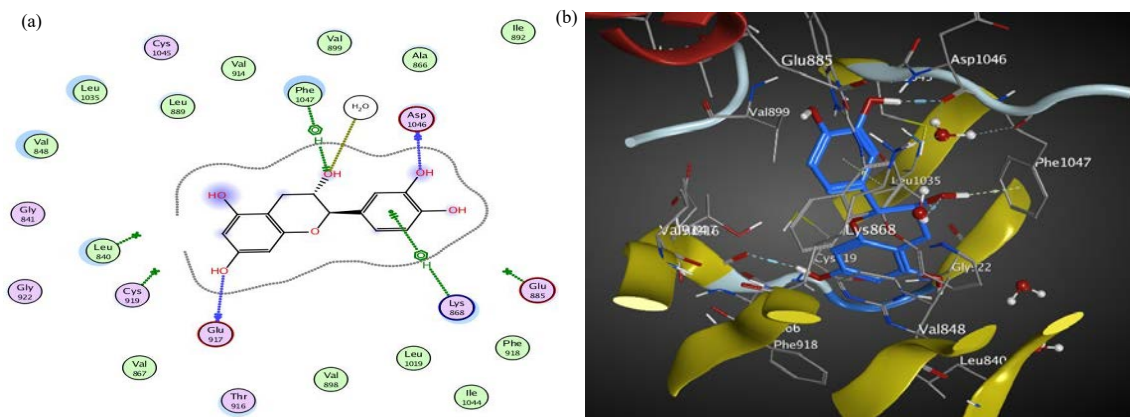


Fig. 3(a-b): (a) 2D and (b) 3D diagram showing the binding mode of catechin (blue) with 3EWH VEGF kinase binding pocket

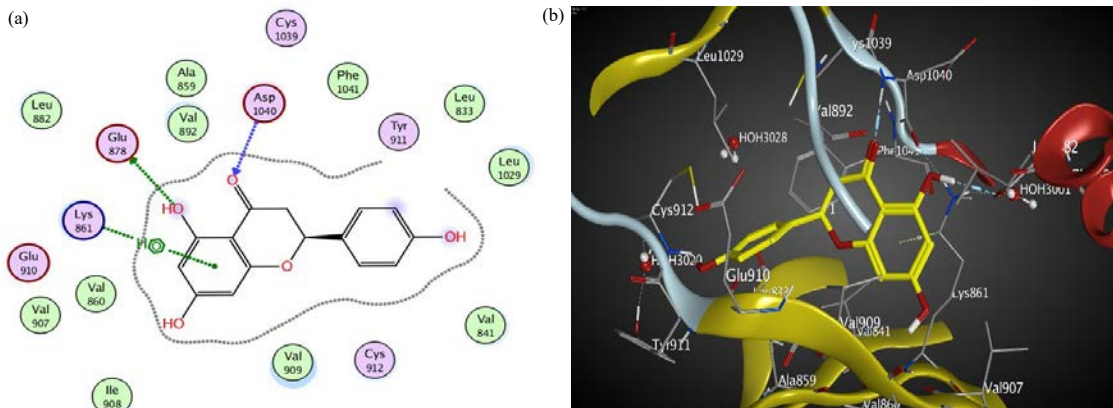


Fig. 4(a-b): (a) 2D and (b) 3D diagram showing the binding mode of naringenin (yellow) with 3HNG VEGF kinase binding pocket

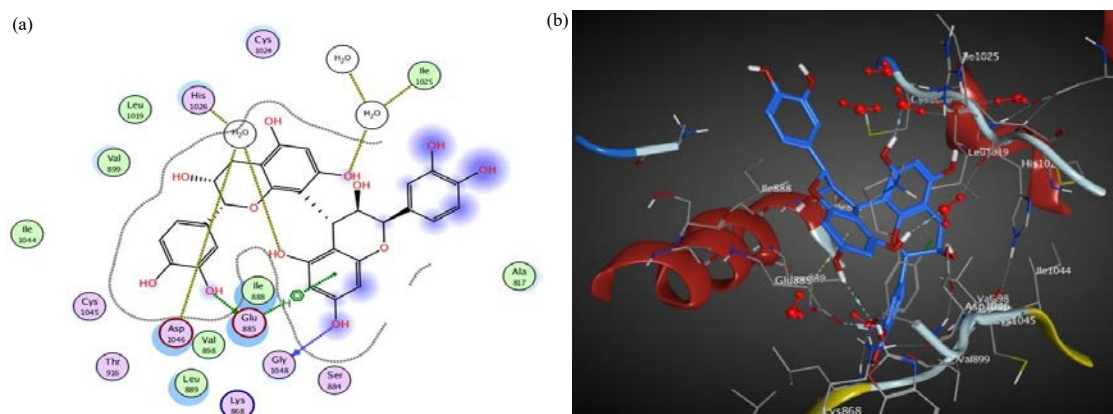


Fig. 5(a-b): (a) 2D and (b) 3D diagram showing the binding mode of procyanidin B2 (blue) with 2QU6 VEGF kinase binding pocket

Table 5: Docked conformations of catechin, naringenin and procyanidin B2 with VEGF kinase proteins

VEGF	Catechin			Naringenin			Procyanidin B2		
	Bonds	Residues	Distance/E (kcal mol <sup>-1</sup> )	Bonds	Residues	Distance/E (kcal mol <sup>-1</sup> )	Bonds	Residues	Distance/E (kcal mol <sup>-1</sup> )
3HNG	3	ILE 1038	3.08/-0.9	3	GLU 878	3.01/-2.6	4	ASP 1040	3.05/-2.3
		CYS 1018	3.19/-0.8		ASP 1040	2.72/-3.4		ILE 1019	2.95/-1.8
2QU6	3	ASP 807	3.48/-0.6	2	LYS 861 (pi-H)	3.69/-0.8	5	THR 877 (pi-H)	3.68/-0.6
		CYS 1024	3.67/-2.3		CYS 919	2.68/-2.8		HOH 1	2.91/ 0.0
		GLU 885	3.05/-3.5		LEU 840	3.64/-0.6		GLU 885	2.66/-2.0
		LEU 889 (pi-H)	4.04/-0.9					HOH 200	2.95/-0.5
2QU5	3	CYS 919	2.64/-0.9	3	PHE 1047 (H-pi)	3.85/-1.3	4	CYS 919	2.72/-2.4
		CYS 919	2.72/-2.8		PHE 1047 (H-pi)	4.51/-0.8		PRO 839	3.24/-1.3
1YWN	2	PHE 1047 (H-pi)	3.69/-0.7	2	CYS 919 (pi-H)	4.23/-0.9	4	LYS 838	2.74/-5.7
		GLU 883	2.68/-0.9		GLU 883	3.16/-0.9		GLY 922 (pi-H)	3.61/-0.6
		GLU 915	2.81/-1.4		ASP 1044 (pi-H)	4.32/-0.7		ASP 1044	2.61/ 1.3
2P2I	3	VAL 899	2.99/-2.8	2	VAL 899	2.79/-3.4	4	ARG 840	2.74/-1.9
		ILE 1025	2.91/-1.4					HOH 127	2.43/ 3.1
		GLU 885	2.80/-4.2		GLU 885	3.03/-1.4		PHE 843 (H-pi)	3.98/-0.6
3EWH	4	ASP 1046	2.66/-2.8	3	HOH 142	2.79/-2.0	2	ARG 1080	2.97/-2.1
		GLU 917	3.06/-2.4		CYS 919	2.91/-2.9		ALA 1050	3.24/-0.9
		PHE 1047 (H-pi)	3.60/-1.2		LEU 840 (pi-H)	3.91/-0.6		ILE 1025	2.90/-1.7
		LYS 868 (pi-H)	4.36/-1.0					TYR1082(pi-H)	4.22/-0.7
								GLU 815	3.16/-2.9
								HOH 183	2.80/-0.6

afatoxin B1-induced carcinogenesis<sup>22,23</sup>. Naringenin has a potentially useful inhibitory effect on tumor growth<sup>24</sup>. Recent studies have shown that procyanidin on human breast<sup>25</sup>, lung<sup>26</sup>, oral<sup>27</sup>, prostate cancers<sup>28</sup> resulted in an inhibition of cell proliferation without a toxic effect on normal cells<sup>29</sup>. VEGF promotes angiogenesis via endothelial cells activation. Procyanidin B2 reduced the expression levels of VEGF and has been reported to alter carcinogen metabolism, inflammation, proliferation, apoptosis and angiogenesis<sup>30</sup>. Thus, VEGF is an ideal anticancer target. The interactions of VEGF-1 and VEGF-2 with catechin, naringenin and procyanidin B2 supported their usage against angiogenic molecular targets. The study demonstrates for the first time that catechin, naringenin and procyanidin B2 are better in inhibiting angiogenesis via inhibiting VEGF.

### CONCLUSION

Tamarind phenolics inhibit cell viability by decreasing angiogenesis. Incorporation of powder of tamarind seeds into the diet significantly increases the bioactive antioxidant and anticancer phytochemicals. Finally; the results may help the scientific community of medicine to carry out lab experiments considering the potential of tamarind seeds.

### SIGNIFICANCE STATEMENT

This study highlighted the potential health benefits of the industrial underutilized by-product of *T. indica* that can be beneficial for consuming as a nutraceutical or food supplement. This study will help the researchers to uncover the critical areas of the anti-angiogenic effect of phenolics that many researchers were not able to explore.

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