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Immunomodulatory and antineoplastic efficacy of common spices and their connection with phenolic antioxidants

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ABSTRACT

Background: Spices have generally offered a conventional way to avert and heal various communicable and non-communicable diseases due to their efficacy and safety and their noteworthy contribution towards understanding targeted drug action and drug delivery systems. Hence, the current investigation is designed to evaluate the immunomodulatory and antineoplastic efficacy of 15 spices that connect with the flavonoid and total polyphenol ingredients. This study includes the 15 adopted spices and their total flavonoid and polyphenol contents, cell viability assay (MTT), immunomodulatory efficacy (NO, TNF-α), and antineoplastic efficacy (using six cancer cell lines).

Methods: The quantification of the flavonoid and phenolic content of methanolic extracts of 15 spices was performed by colorimetric assay. The immunomodulatory efficacy was studied according to their capacity to prevent NO and TNF-α synthesis in LPS stimulated RAW 264.7 macrophages. Cell viability was observed using MTT colorimetric assay. Antineoplastic efficacy was determined with six cancer cell lines, namely liver (HepG2), colon (HT29), breast (MCF7), pancreas (MIA PaCa2), lung (A549) and blood (Raji).

Results: The outcome of significant immunomodulatory efficacy of the spices was noted in the following sequences: *Acorus calamus* L.(Inhibition of NO-49.32 ± 4.29 µg/mL and TNF-α 96.35 ± 8.23 µg/mL)> *Alpinia galanga* Wild (Inhibition of NO-55.69 ± 5.89µg/mL and TNF-α 102.36 ± 8.96 µg/mL)> *Armoracia rusticana* Gaerth (Inhibition of NO-82.44 ± 5.98 µg/mL and TNF-α 115.69 ± 7.59)> *Capparis spinosa* L. (Inhibition of NO-127.59 ± 5.68 µg/mL and TNF-α 123.58 ± 8.56 µg/mL) > *Aframomum melegueta* K. Schum (Inhibition of NO-169.89 ± 6.89 µg/mL and TNF-α 144.59 ± 7.89 µg/mL). The remaining spices considerably inhibited the generation of NO and TNF-α. All spices studied exhibited highly significant antineoplastic effects against all six cell lines. Noteworthy biological activities were observed in *A. calamus, A. galanga, A. rusticana, C. spinose,* and *A. melegueta* which have bulk quantities of polyphenols.

Conclusion: Based on the present findings, spices are possible candidates for novel antioxidant, anti-inflammatory, and antineoplastic agents.

Keywords: Spices; cancer cell lines; immunomodulatory; antineoplastic; total polyphenol contents

INTRODUCTION

Cancer is among one of the dreaded illnesses, possesses morethan 100 diverse categories arising from various molecular alterations located in the cell. Cancer is the second foremost cause of global mortality next to cardiovascular diseases, whereby about 1 in 6 deaths are due to malignancy [1]. Between the years 2007 and 2020, the mortality rates is anticipated to increase by up to 15.2% of males and 8.1% in females [2-4]. In 2019, the office of National Cancer Statistics stated that in the period from 2012-2016, the mortality rates in the poorest nations were double for cervical cancer and 40% higher for pulmonary and liver cancers in men [1, 5]. Although mortality rates are high due to malignancy, several advances have been made with respect to the biology of the illness and treatment [3]. Over the past decade, data suggests that the cancer incidence rate can be reduced based on the opportunity for more reasonable dissemination of effective cancer prevention, early detection, and therapeutic approaches [5].

It is progressively understood that most of the recent diseases are based on the "oxidative stress" which is caused by an imbalance between the oxidizing agents (such as oxygen or hydrogen peroxide) and antioxidant compounds (such as antioxidant enzymes or antioxidant vitamins) in the body. Oxidative stress is normally generated by radicals that pursue steadiness through electron pairing with biological macromolecules (proteins, lipids, and DNA), which cause membrane damage. A free radical is a molecule that contains an unpaired electron in the related atomic orbital. The electron attaches to the structure of the mentioned biomolecules and causes cell damage due to its reactivity and instability [6]. This instability may eventually cause aging, cancer, diabetes, as well as cardiovascular and inflammatory diseases [7-10]. Almost all cells are exposed to oxidative stress, and hence, free radicals and oxidation could play a vital role in carcinogenesis at several tumor spots. Currently, free radical-stimulated oxidative stress and its significance in inflammation and malignancy are well documented [4]. Hence, there is a mutual relationship between free radicals, inflammation, and cancer. The compounds possessing free radical scavenging or antioxidant activities with anti-inflammatory potential are highly valued candidates for anticancer drugs.

This free radical-stimulated oxidative stress is however limited under normal circumstances by defensive systems of the host. These include low-molecular-weight free radical scavengers

(thiols, quinols, ascorbic acid, tocopherols, carotenoids, polyphenols, terpenoids, urate), enzymes that eliminate either oxidants directly (superoxide dismutases) or their precursors (peroxiredoxins, glutathione peroxidases, and catalases that remove peroxides), and several enzyme systems that repair the cellular damage (methionine sulfoxide reductases, disulfide reductases/isomerases, sulfiredoxins) or eliminate impaired cellular materials (proteasomes, lysosomes, proteases, phospholipases, DNA repair enzymes) [9-13]. When an organism is exposed to a high concentration of free radical species, the endogenous antioxidant system is compromised and, thus, fails to assure the complete defense of the host. Due to the lack of these active candidates, the unavoidable expense of existing chemotherapy drugs and their complications, cancer eventually becomes aggressive and can quickly cause mortality. Thus, efforts are being made to pursue the most effective naturally occurring anti-carcinogens that would prevent, reduce, or regress tumor growth.

Spices and medicinal herbs have a distinct place in cancer therapy. Spices have been consumed as food and medicine and are used for flavoring as well as food preservatives for a long time [14]. They are readily present in the household and have been used to treat various illnesses or protect food due to their antimicrobial and powerful natural antioxidant properties [15-17]. They are well known as safe materials for food with insignificant adverse effects. The search for bioactive compounds with effective antioxidants continues to be of great significance in developing remedies against various free radical-mediated diseases. These compounds normally prevent oxidative reactions in cell mitochondria and protect against DNA damage and carcinogenesis. They are promising substances with an extensive series of pharmacological abilities such as anti-inflammatory, anti-bacterial, anti-fungal, and immunomodulatory effects [18-26]. The main spice ingredients considered to have antioxidant properties are polyphenols and terpenes [17, 21]. These secondary metabolites are potential antioxidants due to their oxido-reduction properties [20, 27-29]. Spices are used for the treatment of diseases with a systematic modality constructed based on their antioxidant properties. The random use of herbal medicine is technically not lawful due to the absence of noxious pharmacological host testing. However, the conventional spices do not cause any host toxicity and could be candidates in discovering and developing new anti-inflammatory and antineoplastic agents. Taking this into consideration, the current study aimed to evaluate the immunomodulatory and antineoplastic efficacy of 15 common spices that connect with the antioxidant properties of total phenol and flavonoid contents.

MATERIALS AND METHODS

Chemicals and culture

Lipopolysaccharide (LPS, Cat No. L2887), mouse macrophages (RAW 264.7, Cat. No. 91062702), dimethyl sulfoxide (DMSO, Cat. No. 101900), trypan blue (Cat. No. T6146**)**, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Cat. No. M5655), sulphanilamide (Cat.No.33626), tetra-methyl benzidine, (Cat. No. T5513) and N-(1-1-napthyl) ethylenediamine dihydrochloride (Cat. No. N9125), Dulbecco's modified eagle's medium (DMEM, Cat. No. D6046), fetal bovine serum (FBS, Cat. No. F2442), and antibiotics (Penicillin and streptomycin, cat. No. 516106) were acquired from Sigma Aldrich, MO, USA. ELISA standard kit, TNF-α (Cat. No. 560479), and NO (Cat. No. 23479) were procured from BD Biosciences, San Jose, CA, USA. Six cancer cell lines, namely liver (HepG2), colon (HT29), breast (MCF7), pancreas (MIA PaCa2), lung (A549) and blood (Raji) were obtained from the American Type Culture Collection (ATCC), Manassas, VA, USA.

Preparation of spice extracts

The spices were identified and confirmed with reference to Herbarium sheets available in *The Rapinat Herbarium* and the Centre for Molecular Systematics, St. Joseph's College, Tiruchirappalli, Tamil Nadu, India. Ten grams of a spice powder were solubilized using 100-mL of methanol and kept at 4°C for 72 hours. The composite was then filtered and the filtrate was concentrated to a sticky mass in a rotary evaporator at room temperature; the quantity was calculated and solubilized in 1 mL 10% v/v DMSO; all the spice extracts were kept at 4 °C for further use [16].

Quantification of total phenolic content

The quantity of total phenol was assayed by the Folin-Ciocalteu method using spectrophotometry with minor changes [30]. 2 mL of Folin-Ciocalteu was added with 0.2 mL methanolic spice extract and 5 mL of 20% w/v sodium carbonate. The composite was placed in a dark location at 37°C for at least 30 min and the measurement was read using a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at 765 nm. Total phenolics were calibrated by gallic acid as a standard. All analyses were replicated. Total phenolics are expressed as mg of gallic acid equiv/g of dry mass.

Quantification of total flavonoid content

Total flavonoids were quantified by the method of Stojanović et al. [31] using aluminium chloride ($AlCl_3$). Thereby, 0.5 mL of the spice extract was mixed with 0.5 mL of the reaction medium methanol: water: acetic acid in the ratio of 14:5:1. This prepared solution was added to 4 mL of the $AlCl_3$ reagent and kept at 37°C for 5 min. The absorbance was read using a spectrophotometer at 430 nm. Total flavonoid content was expressed by mg rutin equiv/g dry mass.

Assays for immunomodulatory activity

Activation of RAW 264.7 macrophages

This assay was carried out based on the methods of Ni et al. [32], Zhang et al. [33], and Zhang et al. [34] with slight modifications. RAW 264.7 cells were added to a DMEM medium (containing 1% antibiotic with 5% w/v FBS) and incubated for at least 4-7 days at 37^{o}C in 5% CO_2 to obtain the cell density of 2×10^5 cells/mL.

NO, and TNF-α production

About 100 µL of the supernatant was carefully transferred from the respective well to 6 well plates. 50 µL of sulfanilamide (1% w/v) containing H_3PO_4 (5% w/v) was added to the supernatant and stored for 5 minutes at 37^{o}C. followed by adding 50 µL of Naphthyl ethylenediamine (0.1% w/v) to analyze the generation of NO based on the methods of Ni et al. [32], Zhang et al. [33], and Zhang et al. [34]. Sodium nitrate was used as a standard. The ELISA kit was used to analyze (800™ TS ELISA Absorbance Reader, Bio Tek Instruments, Winooski, VT, USA) the TNF-α concentration as per the procedure of Zhang et al. [35] All analyses were conducted three times.

Cell viability analysis by MTT assay

RAW 264.7 cells are macrophage-like cells that are suitable models of macrophages. The viability of RAW 264.7 cells was determined by performing the MTT colorimetric assay, which is in accordance with the mitochondrial activity. [36] Thereby, RAW 264.7 cells were preserved with spice extracts that were stored at 37°C for 24 h. The supernatant was then discarded and 100 µL of MTT (0.2 mg/mL, dissolved in DMEM) was added to each well and stored for additional incubation for 4 h. The supernatant was removed and 50 µL of DMSO was added to each well to soften the crystal-like formation. The mentioned activity in the living cells is stable and increasing or decreasing the number of living cells is therefore linearly associated with the mitochondrial activity. Tetrazolium MTT staining is activated in the metabolically active cells. Finally, the absorbance was read using colorimetry (Thermo Fisher Scientific, Waltham, MA, USA) at 595 nm. The cell viabilities were measured by the following equation:

$$\text{Inhibition (\%)} = \frac{OD\ sample - OD\ positive\ control}{OD\ negative\ control - OD\ positive\ control} \times 100$$

Antineoplastic assays using six cancer cell lines

All six cancer cell lines were cultivated and protected on the basis of the previous methods with slight modifications [36]. The antineoplastic efficacy was determined using various cancer cell lines, namely liver (HepG2), colon (HT29), breast (MCF7), pancreas (MIA PaCa2), lung (A549) and blood (Raji). The optical density was read using a spectrofluorometer (Jasco FP-8300 Spectrofluorometer, Easton, MD, USA) at 570 nm. DMSO was used as a negative control. The inhibition of different cancer cells (%) was analyzed using the following equation:

$$\text{Cell viability (\%)} = \frac{OD\ sample}{OD\ positive\ control} \times 100$$

Statistical Analysis

The data are given as means ± SD. The result outcomes were matched by a one-way analysis of variance (ANOVA) using SPSS version 22.0 (SAS Institute Inc., Cary, NC, USA). Duncan's multiple range test was employed to find significant changes among the means. The differences at the 5% level ($p < 0.05$) were measured statistically significant.

RESULTS

Ethnomedicinal uses of 15 common spices and their immunomodulatory and antineoplastic efficacy are shown in Table 1. These spices are widely consumed worldwide for culinary and infectious ailments. The results of total flavonoid and polyphenol content of 15 spices are presented in Table 2, which was quantified using aluminium chloride and Folin-Ciocalteu methods, respectively. Flavonoid and polyphenol contents were expressed as mg of rutin-equiv/gm of dry mass and mg of gallic acid-equiv/gm of spice dry mass respectively. The greater contents of total flavonoid and polyphenol content of the five spices were listed based on the order: *Acorus calamus> Alpinia galanga> Armoracia rusticana> Capparis spinosa >A. melegueta*. The highest flavonoid and phenolic contents were observed in the extracts of *Acorus calamus L.* (133.48±10.2 mg/g and 190.5±15.6 mg/g). Usually, the amplified generation of TNF-α and NO produces inflammation. The present study examined the abilities of the 15 spice extracts to inhibit the production of NO and TNF- α in LPS-induced RAW 264.7 cells. The inhibitory activity was measured as CC_{50} values and the results are shown in Table 3.

Table 1 Selected common spices for assay of immunomodulatory and antineoplastic effect [14-17, 26]

S.No	Spices	Family	Common name	Portions used	Ethnomedicinal practices
1	*Acorus calamus* L.	Araceae	Sweet flag	Rhizome	Used to cure inflammation of the stomach lining (gastritis)
2	*Aframomum melegueta* K. Schum.	Zingiberaceae	Grains of Paradise	Seed	Used as a purgative, galactagogue, anthelmintic, and potent anti-inflammatory activity
3	*Alpinia galanga* Wild	Zingiberaceae	Galanga	Rhizome	Used to treat indigestion, colic and dysentery, cancers of mouth and stomach and systemic infections
4	*Anethum graveolens L.*	Apiaceae	Dill seed	Fruits	Used as carminative, stomachic and diuretic and relieve colic pain
5	*Apium graveolens L.*	Apiaceae	Celery	Seed	Used as diuretic, laxative, and sedative and blood cancer
6	*Armoracia rusticana* Gaerth	Brassicaceae	Horse Radish	Root	Used as antiseptic, and value in the treatment of colds, fevers and respiratory and urinary tract infections
7	*Artemisia dracunculus* L.	Asteraceae	Tarragon	Leaf	Stimulates the digestive system and uterus, lowers fevers and destroys intestinal worms
8	Capparis spinosa L.	Capparidaceae	Caper	Flower buds	Used to treat hemorrhoids and gout
9	*Carum carvi L.*	Apiaceae	Caraway	Fruits	Used to dyspepsia, and various spasmodic conditions, bloating, and diarrhoea
10	*Citrus hystrix* DC	Rutaceae	Kaffir Lime Leaves	Leaves	Used against stomach pain, diarrhea, and indigestion
11	*Crocus sativus L.*	Iridaceae	Saffron	Parts of pistil	Useful for asthma, whooping cough and to release phlegm, sleep problems and cancer
12	*Elettaria cardamomum* (L.) Maton	Zingiberaceae	Cardamom	Fruits	Used to cure dysentery, swelling, bronchitis, and cough
13	*Ferula asafetida L.*	Apiaceae	Asafoetida	Resin From rhizome	Used against indigestion, swelling, and toothache
14	*Garcinia indica* Choisy	Clusiaceae	Kokam	Rind	Used in the treatment of inflammatory ailments for rheumatic pains and bowel complaints
15	*Hyssopus officinalis* L.	Lamiaceae	Hyssop	Leaf	Useful for sore throats, colds, hoarseness, and as an expectorant

Table 2. Flavonoid and polyphenolic content of methanolic extracts of 15 spices.

S.No	Spices	Flavonoids (mg RE/g DE)	Polyphenols (mg GAE/g DE)
1.	*Acorus calamus* L.	133.48 ± 10.2^{a}	190.5 ± 15.6^{a}
2.	*Aframomum melegueta* K. Schum.	$95.39 \pm 8.92^{b,c}$	138.36 ± 9.5^{d}
3.	*Alpinia galanga* Wild	125.89 ± 12.3^{a}	173.45 ± 10.9^{b}
4.	*Anethum graveolens L.*	$92.54 \pm 7.49^{b,c}$	99.25 ± 8.9^{e}
5.	*Apium graveolens L.*	78.67 ± 6.95^{d}	85.95 ± 6.4^{e}
6.	*Armoracia rusticana* Gaerth	112.24 ± 11.4^{b}	158.15 ± 12.3^{c}
7.	*Artemisia dracunculus* L.	89.35 ± 6.8^{b}	99.95 ± 9.8^{e}
8.	Capparis spinosa L.	$101.28 \pm 9.65^{b,c}$	144.26 ± 11.2^{d}
9.	*Carum carvi L.*	60.59 ± 5.64^{e}	72.58 ± 5.69^{e}
10.	*Citrus hystrix* DC	75.48 ± 6.25^{d}	86.49 ± 5.98^{e}
11.	*Crocus sativus L.*	59.68 ± 6.58^{e}	72.89 ± 5.21^{e}
12.	*Elettaria cardamomum* (L.) Maton	55.49 ± 5.98^{e}	59.56 ± 4.56^{f}
13.	*Ferula asafetida L.*	85.38 ± 6.27^{c}	89.44 ± 6.48^{e}
14.	*Garcinia indica* Choisy	76.49 ± 5.87^{d}	88.35 ± 4.59^{e}
15.	*Hyssopus officinalis* L.	38.66 ± 4.23^{f}	45.08 ± 3.56^{f}

Results are Means ± SD. Values not sharing a common superscript (a-f) differ significantly at $p< 0.05$, Duncan's Multiple Range Test (DMRT). The quantity of flavonoid is expressed as mg of rutin-equiv/ gm of dry mass and the quantity of polyphenol is expressed as mg of gallic acid-equiv/gm of dry mass. All analyses were conducted triplicate.

Table 3: Immunomodulatory effect of the various spices

Name of the spices	CC_{50} for the inhibition of NO production (µg/mL)*	Cell viability (% of cell survival)**	CC_{50} for the inhibition of TNF-α production (µg/mL)*	Cell viability (% of cell survival)**
Acorus calamus L.	49.32 ± 4.29	108.95 ± 4.26	96.35 ± 8.23	95.26 ± 7.45
Aframomum melegueta K. Schum.	169.89 ± 6.89	86.98 ± 9.15	144.59 ± 7.89	78.95 ± 8.15
Alpinia galanga Wild	55.69 ± 5.89	102.38 ± 4.59	102.36 ± 8.96	93.58 ± 6.32
Anethum graveolens L.	226.84 ± 9.54	83.58 ± 9.25	178.95 ± 7.59	70.25± 6.58
Apium graveolens L.	357.48 ± 11.28	80.26 ± 6.25	324. 59 ± 7.84	58.56 ± 5.49
Armoracia rusticana Gaerth	82.44 ± 5.98	98.74 ± 6.59	115.69 ± 7.59	89.89 ± 7.85
Artemisia dracunculus L.	589.64 ± 15.67	52.35 ± 6.38	554.25 ± 14.56	38.97 ± 5.68
Capparis spinosa L.	127.59 ± 5.68	91.35 ± 8.95	123.58 ± 8.56	83.28 ± 8.45
Carum carvi L.	238.94 ± 9.89	81.36 ± 8.58	298.94 ± 12.35	60.23 ± 5.68
Citrus hystrix DC	338.79 ± 9.56	76.56 ± 9.15	314.67 ± 12.39	59.68 ± 6.32
Crocus sativus L.	229.89 ± 7.14	83.14 ± 8.14	289.25 ± 14.5	64.56 ± 5.79
Elettaria cardamomum (L.) Maton	697.45 ± 12.7	48.68 ± 4.56	559.24 ± 12.5	37.49 ± 4.56
Ferula asafetida L.	456.9 ± 13.47	68.57 ± 7.25	439.78 ± 9.25	41.85 ± 4.67
Garcinia indica Choisy	545.69 ± 18.5	55.69 ± 8.25	512.58 ± 9.57	40.25 ± 5.59
Hyssopus officinalis L.	467.89 ± 9.87	69.25 ± 7.59	416.89 ± 8.45	44.56 ± 6.59

*Inhibition of NO and TNF-α generation was expressed in terms of CC_{50}, $p<0.05$; **cell viability was measured at quantity 1 mg/mL of the spice extract.

Cell viability was measured at 1 mg/mL of the spice extract. It was realized that all spice extracts showed cell viability and noteworthy inhibitory activity against the generation of NO and TNF-α. However, five spices exhibited stronger cell viability and inhibitory activity in the sequence of *A. calamus*> *A. galanga*> *A. rusticana*> *C. spinosa*> *A. melegueta* against the production of NO and TNF-α with CC_{50} values when compared with other spices. *A. calamus* L and *A. galanga wild* showed a significantly higher cell viability rate (108.95% and 102.38%) against NO production. Additionally, *A. calamus L. wild* exhibited significantly higher cell viabilities (95.26%) against TNF-α production. This plant displayed higher inhibitory activity against the generation of NO and TNF-α with CC_{50} values that are less than 49.32±4.29 µg/ mL and 96.35±8.23 µg/ mL respectively. The concentration-dependent (15.625 to 1000 µg/ mL) immunomodulatory activities of most effective extracts from the listed spices are shown in Figures 1a and 1b.

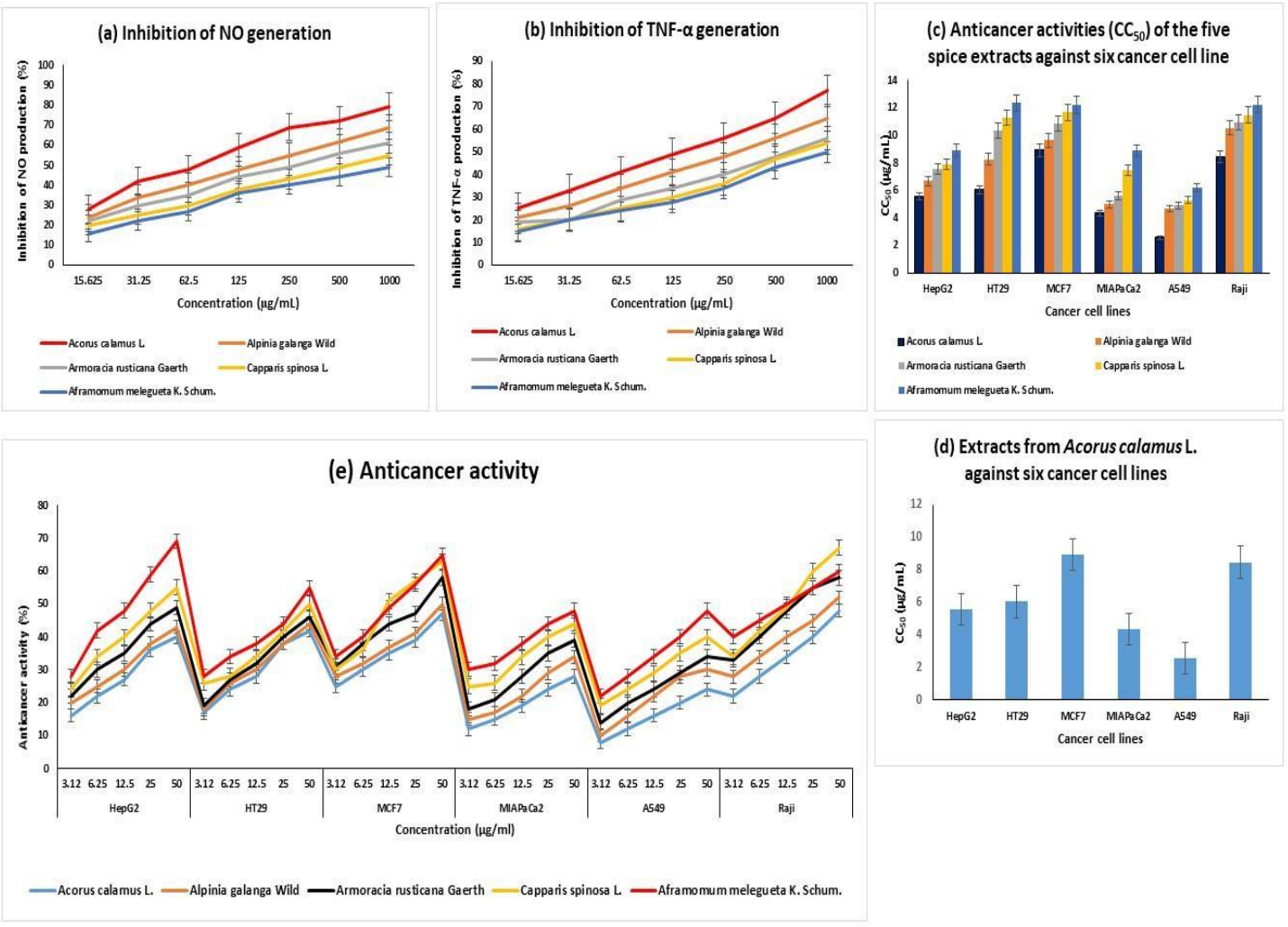

Figure 1: (a) Concentration-dependent immunomodulatory effect (NO generation) of most effective spices from the list; (b) Concentration-dependent immunomodulatory effect (TNF-α generation) of most effective spices from the list (c) Antineoplastic potential (CC_{50}) of the 5 spice extracts against six various cell line (d) Methanolic extracts from *Acorus calamus* L. against six cancer cell lines. (e) Dose-dependent variation of antineoplastic activities of the selected five most active spices. Results are mean ± SD. All analyses were conducted triplicate, $p < 0.05$.

Table 4: Cytotoxicity effect of 15 spices against six cancer cell lines

Name of the spices	CC_{50} (µg/mL)*					
	HepG2	**HT29**	**MCF7**	**MIA PaCa2**	**A549**	**Raji**
Acorus calamus L.	5.56 ± 1.03	6.05 ± 1.89	8.92 ± 1.31	4.35 ± 0.65	2.58 ± 0.65	8.45 ± 4.56
Aframomum melegueta K. Schum.	8.89 ± 1.25	12.35 ± 1.58	12.22 ± 2.35	8.88 ± 1.58	6.22 ± 0.58	12.25 ± 2.58
Alpinia galanga Wild	6.68 ± 0.89	8.26 ± 1.89	9.63 ± 2.45	4.99 ± 0.96	4.65 ± 0.78	10.56 ± 1.36
Anethum graveolens L.	10.68 ± 0.25	14.65 ± 0.89	14.56 ± 2.91	9.25 ± 1.51	10.24 ± 1.35	14.28 ± 2.35
Apium graveolens L.	9.98 ± 0.45	14.44 ± 0.95	15.22 ± 1.58	9.52 ± 1.93	9.58 ± 1.87	19.48 ± 1.53
Armoracia rusticana Gaerth	7.58 ± 5.68	10.35 ± 5.68	10.89 ± 3.25	5.59 ± 1.35	4.89 ± 2.58	10.95 ± 2.01
Artemisia dracunculus L.	17.23 ± 1.34	18.59 ± 0.25	19.89 ± 0.89	18.77 ± 2.55	19.12 ± 1.73	15.59 ± 1.25
Capparis spinosa L.	7.89 ± 3.25	11.28 ± 4.78	11.68 ± 3.68	7.48 ± 1.25	5.28 ± 2.59	11.48 ± 1.28
Carum carvi L.	14.65 ± 0.95	19.52 ± 2.35	18.98 ± 2.55	18.99 ± 2.35	15.26 ± 0.56	16.32 ± 1.25
Citrus hystrix DC	10.98 ± 1.25	17.89 ± 1.89	19.89 ± 2.65	18.23 ± 1.65	18.98 ± 1.25	19.86 ± 2.22
Crocus sativus L.	16.65 ± 0.59	18.44 ± 1.63	19.23 ± 1.62	18.13 ± 1.47	13.25 ± 0.25	15.89 ± 1.05
Elettaria cardamomum (L.) Maton	13.68 ± 2.22	18.98 ± 4.56	14.56 ± 5.47	12.56 ± 2.65	18.25 ± 2.56	15.69 ± 3.32
Ferula asafetida L.	16.58 ± 5.68	22.35 ± 5.68	16.89 ± 3.25	10.59 ± 1.35	14.89 ± 2.58	12.35 ± 2.01
Garcinia indica Choisy	10.25 ± 1.35	18.87 ± 2.25	12.56 ± 1.29	10.67 ± 2.91	10.12 ± 1.54	13.65 ± 0.98
Hyssopus officinalis L.	15.56 ± 2.03	16.05 ± 5.89	18.92 ± 2.31	14.35 ± 2.65	12.58 ± 3.65	18.45 ± 4.56

* Smaller CC_{50} value indicates higher cytotoxicity activity, Mean ± SD

All spice extracts had noteworthy higher immunomodulatory activity. Among them, *A. calamus L.* had maximum inhibitory activities against NO as well as TNF-α production. The upright relationship between the immunomodulatory effects of the spices and their flavonoid and polyphenol contents shown in Table 5 were fascinating results.

Notably, *A. calamus, A. galanga, A. rusticana, C. spinosa,* and *A. melegueta* had remarkable inhibition in the generation of NO as well as TNF-α (in terms of lower CC_{50} values) and also had maximum quantities of flavonoid and total phenol. Other spices were identified to contain low quantities of flavonoid and total phenol that showed less immunomodulatory effects (Tables 2 and 3). The antineoplastic efficacy of the extracts from 15 spices was evaluated in six cancer cell lines including liver (HepG2), colon (HT29), breast (MCF7), pancreas (MIA PaCa2), lung (A549) and blood (Raji). These results are shown in Table 4. It is interesting that all listed spices had a potential cytotoxic effect that significantly inhibited growth in all six cancer cell lines. The correlation of antineoplastic efficacy of spices and their flavonoid and polyphenol quantities are presented in Table 4. Notably, five spices demonstrated stronger cytotoxicity activity in the sequence of *A. calamus> A. galanga> A. rusticana> C. spinosa> A. melegueta* against six cancer cell lines with lower CC_{50} values when compared with other spices (Figure 1c). Normally, the smaller CC_{50} value

showed higher cytotoxicity activity. During this investigation, *A.calamus L.* had maximum antineoplastic activities against the following sequence of cancer lines: lung> pancreas>liver>colon>blood>breast (Figure 1d). Dose-dependent (3.12 to 50 µg/ mL) antineoplastic activities of the most effective extracts from the listed spices are shown in Figure 1e.

All spice extracts had noteworthy higher antineoplastic activities against all six cancer cell lines. Spices examined in this study demonstrated a substantial correlation of antineoplastic activities with their flavonoid and phenolic contents. Notably, *A. calamus, A. galanga, A. rusticana, C. spinosa,* and *A. melegueta* contained maximum to medium quantities of flavonoid and polyphenols that exhibited significant antineoplastic activities (Table 5).

Table 5: Antineoplastic spices identified in the investigations together with the total flavonoid and polyphenol contents.

Spices	Total flavonoid contents	Total polyphenol contents	Immunomodulatory activity	Antineoplastic activity	Comments
Acorus calamus L.	133.48 ± 10.2^{a}	190.5 ± 15.6^{a}	++	++	Highly potential candidate
Aframomum melegueta K. Schum.	$95.39 \pm 8.92^{b,c}$	138.36 ± 9.5^{d}	++	++	Highly potential candidate
Alpinia galanga Wild	125.89 ± 12.3^{a}	173.45 ± 10.9^{b}	++	++	Highly potential candidate
Anethum graveolens L.	$92.54 \pm 7.49^{b,c}$	99.25 ± 8.9^{e}	+	+	Potential candidate
Apium graveolens L.	78.67 ± 6.95^{d}	85.95 ± 6.4^{e}	+	+	Potential candidate
Armoracia rusticana Gaerth	112.24 ± 11.4^{b}	158.15 ± 12.3^{c}	++	++	Highly potential candidate
Artemisia dracunculus L.	89.35 ± 6.8^{b}	99.95 ± 9.8^{e}	+	+	Potential candidate
Capparis spinosa L.	$101.28 \pm 9.65^{b,c}$	144.26 ± 11.2^{d}	++	++	Highly potential candidate
Carum carvi L.	60.59 ± 5.64^{e}	72.58 ± 5.69^{e}	+	+	Potential candidate
Citrus hystrix DC	75.48 ± 6.25^{d}	86.49 ± 5.98^{e}	+	+	Potential candidate
Crocus sativus L.	59.68 ± 6.58^{e}	72.89 ± 5.21^{e}	+	+	Potential candidate
Elettaria cardamomum (L.) Maton	55.49 ± 5.98^{e}	59.56 ± 4.56^{f}	+	+	Potential candidate
Ferula asafetida L.	85.38 ± 6.27^{c}	89.44 ± 6.48^{e}	+	+	Potential candidate
Garcinia indica Choisy	76.49 ± 5.87^{d}	88.35 ± 4.59^{e}	+	+	Potential candidate
Hyssopus officinalis L.	38.66 ± 4.23^{f}	45.08 ± 3.56^{f}	+	+	Potential candidate

Results are Means ± SD. Values not sharing a common superscript (a-f) differ significantly at $p< 0.05$, Duncan's Multiple Range Test (DMRT). The quantity of flavonoid is expressed as mg of rutin-equiv/ gm of dry mass and the quantity of polyphenol is expressed as mg of gallic acid-equiv/gm of dry mass. ++: exceptionally maximum activity or bulk quantities of polyphenols; +significant activity or significant quality of polyphenol.

The symbolization "double plus" is employed to signify an exceptionally high effect or bulky masses of total polyphenols in the spices. The note "single plus" is aimed to denote a noteworthy activity or a notable mass of polyphenols. Table 5 shows that the methanolic extracts of spices,

specifically *A. calamus***,** *A. galanga, A. rusticana***,** *C. spinosa*, and *A. melegueta* showed exceedingly significant effects and also comprise large amounts of total flavonoids and polyphenols.

DISCUSSION

Over the last decade, spices and medicinal herbs have been valued and recognized as medicinal sources worldwide and have a greater influence on both international health and trade. Therefore, spices and therapeutic herbs continue to play a significant function in the pharmaceutical and healthcare system of the global population [37, 38]. The conventional system of medicine is broadly used as Complementary and Alternative Medicine (CAM) in India, China, Korea, Africa, Europe, Iran, and other parts of the world, which has a substantial quantity of investigation on medicinal chemistry, pharmacognosy, pharmacology, and clinical therapeutics. CAM is the term for medical products and practices that are used along with standard medical treatments practiced by several health professionals [39]. The herbal products have been consumed as "dietary supplements" that have been with various vitamins, and minerals, intended to complement the diet, which potentially provide a positive effect on health beyond basic nutrition as functional foods [40]. Indeed, there are several spices and medicinal herbs being consumed for the inhibition and therapeutic effect on inflammation and cancer [41]. Nevertheless, merely a limited number of herbs have attracted the attention of researchers to explore the medication for tumor or cancer (neoplasm).

Various complications of the currently used anti-inflammatory and antineoplastic drugs have ensued in either withdrawal or reduction of these medications from the pharmacological market. It is fascinating to explore that certain spices and medicinal herbs preferentially inhibited the generation of TNF-α and NO, which is believed to be accountable for modulating inflammation besides their crucial role in immune-inflammatory response [42]. These inflammatory substances are also recognized to cause cell death and tissue injury since NO can respond with free radicals (superoxides) to yield peroxynitrite that can lead to permanent damage to cell membranes [6, 13]. Production of these NO causing membrane injury ultimately leads to cancer formation [41]. This outcome specifies the implication of certain spices and medicinal herbs as possible candidates for effective and safe anti-inflammatory/anticancer agents. Chronic inflammation plays a greater role in the burdens related to pathological conditions in both advanced and emerging nations. Studies have also exposed that phenolics and flavonoids act as exceptional anti-inflammatory agents [35].

The anti-inflammatory properties of flavonoids have been widely studied and favorable effects have been demonstrated in many animal models [36, 43]. Inhibition of NO is not considered a general feature of plant flavonoids and phenolic compounds. However, these compounds have been reported to inhibit NO production, thereby downregulating the expression of NO [36]. In this study, *A. calamus, A. galanga*, *A. rusticana, C. spinosa*, and *A. melegueta* exhibited significant concentration-dependent inhibitory effects counter to the generation of NO and TNF-α with lower CC_{50} values.

All spice extracts presented significant cell viabilities (higher in *Acorus calamus L.* - 108.95% and lower in *Elettaria cardamomum* (L.) Maton - 48.68%). These outcomes suggest that the methanolic extracts of spices express low toxicity and these results coincide with the previous studies that display the least toxicity effects [33, 34, 36, 41, 43]. The results of excess production of NO may lead to the initiation and progression of cancer formation [44-46]. In this circumstance,

agents that inhibit the generation of NO are valuable. In our study, all spices exhibited the maximum inhibitory effect against the generation of NO and these outcomes strongly acclaim that the spice extracts possess anti-inflammatory substances. Previous studies reveal that polyphenols are a major class of anti-inflammatory constituents [11, 27-29]. The correlation between the greater anti-inflammatory effects and total flavonoid and phenolic contents of the spices are shown in **Table 5**. The presentable results are consistent with the earlier reports that the quantities of total flavonoid and phenolic compounds show anti-inflammatory activities [6, 47, 48]. In respect to total flavonoid and polyphenols, phenolic acids (acetosyringone, 4-hydroxybenzoic acid, syringaldehyde, cinnamic acid, caffeic acid, ferulic acid, and tannic acid) were isolated from *Acorus calamus* [49], kaempferol and quercetin from *Armoracia rusticana* [50], quercetin encompass the huge amount in *Capparis spinosa* [51], 1′-acetoxychavicol acetate in *Alpinia galangal* [52], and eugenol in *Aframomum melegueta* [53]. Therefore, it is resolved that the chemical constituents of these polyphenol/flavonoids may be responsible for the anti-inflammatory properties.

Continued oxidative stress can lead to inflammation and tissue injury that may be a possible root cause of tumor development and progression [54]. Hence, the representatives/substances that concurrently have antioxidant, anti-inflammatory and antineoplastic ingredients are beneficial for the inhibition and management of cancer [1, 4]. Fifteen spices examined in the present investigation showed biochemical activities and thus all listed spices are highly appropriate candidates for anticancer medications. Flavonoids in particular and polyphenols, in general, are recognized in the previous investigations to be extremely possible anti-tumorigenic agents [3, 28, 29, 54-56]. All spices inspected in this investigation indicated a noteworthy correlation of anticancer activities with their total flavonoid and phenolic quantities. *A. calamus***,** *A. galanga*, *A. rusticana***,** *C. spinosa*, and *A. melegueta* exhibited maximum to medium quantities of flavonoids and polyphenols and demonstrated noteworthy anticancer effects.

For instance, phenolic compounds such as gallic acid and protocatechuic acids that are normally present in nature have attained more consideration due to their superior pharmacological properties [57, 58]. Both phenolics have been proved to exhibit dose-dependent cytotoxicity on prostate (PC-3) and breast (MCF-7) cancer cells [59]. Mechanistically, gallic acid inhibited the histone deacetylase enzyme thus modifying the acetylation process thereby inducing the cancer cell death [60]. Protocatechuic acid targets the RhoB activation leading to decreased MMP-2 production thus inhibiting cancer cell migration [58]. These compounds induce apoptosis and enhance lactate dehydrogenase levels by reducing the mitochondrial membrane potential. In addition, these compounds trigger the fragmentation of DNA in breast, lung, liver, and prostate cancer cell lines [61]. Similarly, gallic acid inhibits cancer cell proliferation by promoting the generation of reactive oxygen species and arresting cells in the G2/M phase [62]. Similarly, another phenolic compound, caffeic acid, which is also a well-known antioxidant, reported modulating key signaling pathways such as NF- kβ, MAPK and AKT [63]. Furthermore, caffeic acid induced cell death via apoptosis and cell cycle arrest in cell lines representing carcinomas of the oral cavity, neck, and tongue [64].

Thus, it can be concluded that the antineoplastic activity exerted by these spice extracts could be due to the presence of phenolic acids. However, the utility of crude extracts comprising a mixture of phenolic compounds as medicines requires comprehensive studies as the quantity of each phenolic compound in the extract might differ from source to source as well as from the method of extraction and fractionation.

CONCLUSION

In the present study, a novel correlation of the immunomodulatory and antineoplastic effects with the polyphenol contents of 15 spices was presented. *A. calamus, A. galanga, A. rusticana, C. spinosa*, and *A. melegueta* are acknowledged in this investigation as significant candidates for the sighting of novel antineoplastic agents. This immunomodulatory and antineoplastic effect of the spices is due to the presence of phenolic and flavonoid composition which hass a relatively high antioxidant potential as well as the ability to combat oxidative stress associated with cancer. It is, therefore, concluded that these five spices are an extremely potent source of bioactive compounds for the discovery of novel antineoplastic agents, suggesting its use in medicine and food industries.

List of Abbreviations: AKT- serine/threonine-specific protein kinase, $AlCl_3$ - aluminium chloride, ANOVA- analysis of variance, ATCC- American type culture collection, CAM- complementary and alternative medicine, CC_{50}- cytotoxic concentration, DMEM- Dulbecco's Modified Eagle Medium, DMSO- dimethyl sulfoxide, DNA- deoxyribonucleic acid, ELISA- enzyme linked immunosorbent assay, FBS- fetal bovine serum, H_3PO_4- phosphoric acid, LPS- lipopolysaccharides, MAPK- mitogen-activated protein kinase, MMP-2- Matrix metalloproteinase-2, MTT- 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide), NF-kβ- nuclear factor kappa B, NO- nitric oxide, OD- optical density, SPSS- statistical package for the social sciences, TNF-α -tumor necrosis factor-alpha.

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