World Journal of Pharmaceutical Sciences
ISSN (Print): 2321-3310; ISSN (Online): 2321-3086
Published by Atom and Cell Publishers
Available online at: http://www.wjpsonline.com/
Research Article

Inhibitory effect of phenolic extract of *carum carvi* on inflammatory enzymes, hyaluronidase and trypsin

N. B. Thippeswamy[1] and Rajeshwara N. Achur[2]*

[1]Department of Microbiology and [2]Department of Biochemistry, Kuvempu University, Shankaraghatta, Shimoga 577 451, Karnataka, India

Received: 18-12-2013 / Revised: 28-12-2013 / Accepted: 18-03-2014

ABSTRACT

Inflammation is initiated as a healing process by the tissue in response to an injury due to pathogens, irritants, or cell damage. Serine proteases and hyaluronidases are the common cellular enzymes involved in the process of inflammation. Dietary polyphenols have been found to inhibit inflammatory enzymes and reduce the risk of inflammatory diseases. In the present study, we have determined the inhibitory activity of phenolic extract isolated from caraway (*Carumcarvi*) seeds on hyaluronidase and trypsin. The proximate composition of caraway seeds and the composition of phenolic extract have also been determined qualitatively and quantitatively.The data indicates thatcaraway seedsare rich in carbohydrates and fiber and the major phenolic compounds were found to be caffeic acid (475µg/g) and ferulic acid (350µg/g) together with other polyphenols. The phenolic extract significantly inhibited the activities of hyaluronidase and trypsin with an IC_{50} value of 336µg/mL and 46µg/mL, respectively. The various phenolic compounds present in the extract of carawaysynergistically inhibitthe activities of hyaluronidase and trypsin, which may be exploited for the treatment of inflammatory diseases.

Keywords: Inflammation, phenolic compounds, hyaluronidase, trypsin, caraway, glycyrrhizin, ovomucoid

INTRODUCTION

The *Carumcarvi*,commonly called as caraway and its dried ripe fruits are used in folk medicine as a carminative, found to be effective against spasmodic gastrointestinal complaints, flatulence, irritable stomach, indigestion, lack of appetite, dyspepsia in adults and in relieving flatulent colic of infants [1-4]. In Moroccan traditional medicine, the aqueous extract of caraway is used as diuretic, spasmolytic and gastric stimulant [5]. The plant extract and the volatile oils from caraway have also been used as an antiulcerogenic agent [6]. Furthermore, experimental studies have shown its anti-tumor [7], anti-proliferative [8],and anti-hyperglycemic [9] activities.

Inflammation is body's response to disturbed homeostasis caused by infection, injury, or trauma, resulting in systemic and local effects. Many types of cell injury can cause inflammation, including hypoxia, burns, drugs, infectious agents and immunologic reactions[10]. The inflammatory response is a complex self-limiting process precisely regulated to prevent extensive damage to the host. Many diseases are the manifestation of chronic inflammation such as, rheumatoid arthritis asthma and gastrointestinal diseases[11]. Many enzymes have been reported as mediators of inflammation and seem to be involved in both acute and chronic inflammatory disorders [12]. It is well known that serine proteases and hyaluronidases are the common mediators in the process of inflammation. These enzymes are increased by neutrophil stimulation in a variety of inflammations and hypersensitivity-based human diseases [13-15].Therefore inhibition of hyaluronidaseand trypsin may be crucial in reducing disease progression of allergies and inflammation.

Currently, many of the anti-inflammatory drugs are in use but their continuous administration leads to adverse side effects [16, 17]. Therefore, there is a need to explore alternative components to lower the formation of inflammatory mediators with the help of natural dietary products. Natural products have long been contributed to the development of

***Corresponding Author Address:** Dr. Rajeshwara N. Achur, Department of Biochemistry, Kuvempu University, Shankaraghatta- 577 451, Shimoga, Karnataka, India; Email:rajachur@gmail.com*

modern therapeutic drugs [18]. Multiple studies, both epidemiological and experimental, suggest that polyphenols possess anti-inflammatory and antioxidant activity that may contribute, via the diet, to the prevention of chronic diseases such as cancer, cardiovascular disease, inflammatory bowel disease, and Alzheimer's disease[19-23]. Dietary polyphenols have been found to inhibit cellular enzymes such as 5-lipoxygenase, hyaluronidase and trypsin, thus exerting an important anti-inflammatory action [24-26]. The aim of the present study was to determine the composition of phenolic compounds in thecaraway phenolic extract and measurement of inhibitory activity on hyaluronidase and trypsin.

MATERIALS AND METHODS

Caraway seeds and chemicals: The caraway seeds were obtained from the supermarket located in Ontikoppal, Mysore, Karnataka, India.Arachidonic acid, phenidone, NDGA,hyaluronidase, sodium hyaluronate, glycyrrhizin, trypsin, *N*-benzoyl-D-L-arginine-*p*-nitroanilide(BAPNA),ovomucoidand standard fatty acid esters and phenolic compounds namelygallic acid, catechuic acid, caffeic acid, cinnamic acid, ferulic acid quercetin and kaempferolwere obtained from Sigma chemicalsco., St. Louis, MO, USA.All the solvents and other chemicals used in this study were of analytical grade and obtained from Hi Media, Mumbai, India.

Determination of proximate composition

Moisture: The moisture content of caraway seeds was determined according to the standard method of AOAC. The sample (2g) was weighed in analuminiumdish and placed in a hot air oven maintained at 130 ± 1ºC for 4h and was then cooled to room temperature in a desiccator and the weight loss in percentage was reported as moisture content of the seeds.

Carbohydrate: The carbohydrate content of caraway seeds was estimated by phenol-sulphuricacid method [27]. The sample (0.5mL)was mixed with 0.3mL of 5% phenol to which 1.8mL of concentrated sulphuric acid was added and mixed thoroughly. After 20min of incubation at ambient temperature, the absorbance was measured at 480nm using a spectrophotometer. The carbohydrate content was determined by referring to the standard graph prepared using D-glucose (0-50µg/0.5mL).

Protein: The protein content of caraway seeds was estimated by micro-Kjeldahl method according to the method of AOAC. The sample (1g) was digested with concentrated H_2SO_4 (20mL) in the presence of catalytic mixture (98 parts of potassium sulphate and 2 parts of copper sulphate) till the solution became clear. The contents of the flask were cooled and the volume was made up to 100mL with water in a volumetric flask. The 5mL of the digested material was steam distilled in presence of 10mL of 40% NaOH. The liberated ammonia was absorbed into a container containing 10ml of 2% boric acid and a few drops of methyl red indicatorwere added. This solution was titrated with 0.01N HCl, till it became bluish green. Simultaneously, a running blank was processed as above, with water in the place of sample. Ammonium sulphate solution was used as the standard to estimate the amount of nitrogen content of the sample.

Lipid: Total lipid content of caraway seeds was determined according to method of AOAC. 10g of the powdered caraway seeds was packed in a thimble and extracted with 200mL of hexane in a Soxhlet's apparatus at 60ºC for 16h. The extract was transferred to a previously weighed, dry flat bottom flask and solvent was evaporated over hot water bath. The flask was dried, cooled and final weight was taken. The fat content was expressed as g/100g of sample (percentage).

Ash: The ash content of caraway seeds was determined by gravimetric method according to the procedure described by AOAC. 10g of caraway seeds was weighed in a clean silica crucible and heated in a muffle furnace for 5 h at 550ºC and the crucible was cooled in a desiccator. The weight of the ash was determined and expressed as percentage of original sample.

Extraction of polyphenolic compounds from caraway: Carawayseeds were finely powdered in a mixer grinder and the powder was defatted using hexane in a Soxhlet's apparatus for 6h. 10 g of defatted powder was extracted with 100ml of 1:1 ratio of 70% aqueous acetone and 70% aqueous methanol by stirring for 2h. The residue was extracted thrice with the same solvent mixture. All the extracts were combined and concentrated under vacuum in a rotavapor and subjected to hydrolysis with 2N HCl for 30 minutes to facilitate the breakage of glycosides. It was then phase-separated with hexane to remove any traces of fatty acids and subsequently with ethyl acetate to extract polyphenoliccompounds. The ethyl acetate phase was concentrated under vacuum and the concentrated extract was kept at 4ºC until use.

Estimation of total phenolic compounds: The total phenolic content of the extract was estimated by Folin-Ciocalteaumethod[28]. Appropriate

dilutions of phenolic extract was added to 2ml of 2% sodium carbonate solution followed by 100µl 50% Folin-Ciocalteau solution. After keeping the mixture for 30min, the absorbance was measured at 750nm. The phenolic content was expressed as gallic acid equivalents (GAE) per mg of extract calculated from standard graph of gallic acid.

Separation and identification of phenolic compounds by HPLC and LC-MS

HPLC: Phenolic extract of caraway was dissolved in methanol and subjected to HPLC for qualitative and quantitative analysis.The HPLC system (Shimadzu LC-10 A, Japan) is equipped with dual pump LC-10AT binary system, UV detector SPD-10A, Phenomenex Luna reverse phase C_{18} column (i.d. 4.6 mm × 250 mm) and the data was integrated by Shimadzu Class VP series software. The mobile phase consisted of (A) 2% acetic acid in water and (B) acetonitrile. The gradient programme for HPLC was 20% B at 0 min, 30% at 15.0 min and finally to 60% at 40.0 min. The amount of phenolic compounds was calculated by comparison of peak area (254 nm) of the individual phenolic compounds with that of standards. Known quantities of phenolic compound standards such as caffeic acid, cinnamic acid, ferulic acid, gallic acid, catechuic acid, quercetin and kaempferol were used for the identification and quantification of phenolic compounds present in the extract of caraway seeds.

HPLC-ESI-MS: An API 200 triple quadrupole mass spectrometer was used for determining the mass of the phenolic compounds. Analyses were performed on a Turbo ions spray source in negative mode by using nebuliser gas (N_2), focusing potential −400 V, entrance potential −10, declustering potential (DP) 25–60 and collision energy (CE) 15–35. Full scan acquisition was performed by scanning from *m*/*z* 150–700u at a cycle time of 2s. MS product ions were produced by collision-associated dissociation (CAD) of the selected precursor ions in collision cell. In all experiments, both the quadrupoles (Q_1 and Q_2) were operated at unit resolution. Product ion scan of selected molecules were carried out in order to confirm the structure of compounds.

Inhibitory effect of caraway phenolic extract on hyaluronidaseactivity: The inhibitory effect of caraway phenolic extract on activated hyaluronidase (EC 3.2.1.35) (hyaluronoglucosaminidase) was determined by the modified method described by Asada *et al*[29]. Hyaluronidase was activated by incubating 100 µl hyaluronidase (4.15 mg/mL in 0.1 M acetate buffer, pH 3.8) with 50 µl sodium chloride (26.3 mg/mL in 0.1 M acetate buffer pH 3.8) for 20 minutes at 37°C. Following activation, the enzyme mixture was pre-incubated with 200 µl of test samples/reference standard at various concentrations for 20 minutes at 37°C. After pre-incubation, 150µl of sodium hyaluronate (6mg/mL in 0.1 M acetate buffer pH 3.8) was added and the reaction mixture was incubated at 37°C for 40 minutes. The reaction was arrested by the addition of 0.1 mL (0.4 N) of sodium hydroxide and 100 µl (0.8 M) potassium tetraborate. The mixture was kept in boiling water bath for 3 minutes, cooled to room temperature and 3 mL of 67mM DMAB (p-dimethyl amino benzaldehyde) was added and incubated at 37°C for 20 minutes. The absorbance was measured at 585 nm and controls were run which are devoid of test samples.

Inhibitory effect of caraway phenolic extract on the activityof trypsin: Trypsin (EC 3.4.21.4) inhibition was determined by a modifiedmethod of Cannel *et al*.,[30]. Trypsin was dissolved in 50 mMTris-HCl, pH 7.6, to a concentration of 150 units/mL.*N*-benzoyl-D,L-arginine-*p*-nitroanilide (BAPNA, 4.6 mg) was dissolved in 100 µL DMSO and used as substrate. The sample containing 400µL of 0.4M Tris-HClbuffer of pH 7.5, 400µL of enzyme solution,and 800µL of test solution / reference standard of different concentrations were pre-incubated at 37°C for 30 min. After pre-incubation, 800µL of substrate solution was added and incubated at 37°C for 1h. The control reaction was carried out without the test sampleand the absorbance was read at 410 nm.

Statistical analysis: All the experimental data were presented as the mean ± standard error of the mean of three individual measurements. The differences in mean values were tested using one-way analysis of variance (ANOVA) to determine the significant differences among the test materials. The differences were considered to be significant at $p \leq 0.05$.

RESULTS

Caraway seed powder was used for determining the proximate composition and defatted seed powder for profile of phenolic compounds. The phenolic compounds in the phenolic extract were separated and quantified by reverse phase HPLC coupled with mass spectrometry.

Proximate composition of caraway seeds: The proximate composition of caraway seeds is presented in Table 1. The data indicates that the caraway seeds are good source of carbohydrate and fiber. The carbohydrate content was 50.1% and the fiber content was 25.3%whereas the amount of

protein, lipid and ash were foundto be 7.6%, 8.7%, and 4.1%, respectively.

Isolation and identification of phenolic compounds by HPLC and LC-MS: The polyphenolic compounds from defatted powder of carawayseeds were extracted with equal volume of methanol (70%) and acetone (70%) to facilitate extraction of variety of polyphenols. By using this solvent system, we could extract a number of phenolic acids and flavonols from caraway. In our previous studies, we have shown that the phenolic content was 50.20mg/g of defatted powder measured as gallic acid equivalent (GAE) [31].

The identification and quantification of individual phenolic compound was achieved by comparing retention time and the peak area of phenolic compoundspresent in the extract with that of standards at 254 nm. Interestingly, caraway contained a mixture of phenolic acids including gallic acid, catechuic acid, caffeic acid, cinnamic acid, ferulic acid and flavonols such as quercetin and kaempferol (Table 2). Quantitatively, different phenolic acids in caraway extract were found to be 0.475mg of caffeic acid/g, 0.350mg of ferulic acid/g, 0.148 mg of gallic acid/g, 0.125mg of cinnamic acid/g and 0.105mg of catechuic acid/g. Further, two flavonoid compounds were estimated to be 0.129mg of quercetin/g and 0.69mg of kaempferol/g (Table. 2). The structures of phenolic compounds were further confirmed by LC-MS. The LC-MS characteristics of identified phenolic compounds are given in Table 3.

Inhibitory effect of caraway phenolic extract on hyaluronidase: The inhibitory effect of caraway phenolic extract was determinedat different concentrations(25-400µg/mL) on the activity of inflammatory enzyme hyaluronidasein comparison with glycyrrhizin, a synthetic inhibitor as shown in Figure 1.The phenolic extract exhibited dose dependent inhibitory response in the concentration range of 25-400µg/mL and the activity was comparable to that of the synthetic inhibitor glycyrrhizin. The IC_{50} value for the caraway phenolic extract was 336µg/mL, whereas the IC_{50} value for glycyrrhizin was found to be 271µg/mL.Thus the inhibitory activity of caraway phenolic extract was comparable to that of the synthetic inhibitor, glycyrrhizin.

Inhibitory effect of caraway phenolic extract on trypsin: The inhibitory activity of caraway phenolic extract was also tested at different concentrations(5-170µg/mL) on a serine protease enzyme, trypsin,in comparison with ovomucoid, a synthetic inhibitor. The carawayphenolic extract exhibited dose dependent inhibitory responseon trypsin as shown in Figure 2.Caraway phenolic extract inhibited the enzyme with an IC_{50} value 46µg/mL, whereas the IC_{50} value of ovomucoid was found to be 21µg/mL.

DISCUSSION

In the present study, we focused onthe determination of proximate composition,separation and characterization of phenolic compounds from caraway and their biological effects on inflammatory enzymes,hyaluronidase and trypsin. The proximate composition of caraway seedswas estimated and found to be rich in carbohydrates (50.1%) and fiber (25.3%) and relatively less quantity of moisture, protein, lipids and ash. The phenolic compounds present in the phenolic extract of caraway were separated and identified by reverse phase HPLC. Interestingly, carawayhas seven different phenolic acids with varied concentration including gallic acid, catechuic acid, caffeic acid, cinnamic acid, ferulic acid and also flavonols such as quercetin and kaempferol. Caffeic and ferulic acids were found to be most abundant among phenolic acids and quercetin was found to be the main flavonolpresent in caraway seeds.Based on this data, the concentration of various phenolic compounds identified from phenolic extract of caraway is in the order caffeic acid>ferulic acid>gallic acid >quercetin>cinnamic acid >catechuic acid >kaempferol. The data thus indicates that caraway seeds are good source of variety of phenolic compounds. The caraway phenolic extract exhibited dose dependent inhibitory responseon hyaluronidase and the activity was at microgram level with an IC_{50} value of 336µg/mL.However, it was less potent as compared to that of synthetic inhibitor, glycyrrhizin. The literature indicates that polyphenolic compounds such ascurcumin and tannic acid are good inhibitors of hyaluronidase with an IC_{50} value of 57µM and 86µM, respectively[32]. From the data, it is clearly evident that the phenolic extract of caraway is a highly potentinhibitor ofhyaluronidaseactivity at relatively very less concentration as compared to curcumin and tannic acid. The proteolytic enzymes and proteins also play an essential role in inflammation and other functions of the immune system. An earlier report indicates that phenolic compounds and flavonoids to be a competitive inhibitor of serine protease such as trypsin[33]. In the present study, the inhibitory activity of caraway phenolic extract was tested on trypsin and compared withovomucoid, a synthetic inhibitor. The caraway phenolic extract exhibited dose dependent response and inhibited the activity of trypsin at microgram level with an IC_{50} value 46µg/mL.The inhibitory activity of caraway phenolic extract thus showed

less potency as compared to that of ovomucoidwhich is a synthetic inhibitor.Although the inhibitory activity of caraway phenolic extract is less, it could be preferred over ovomucoidas being a natural product.

CONCLUSIONS

The data presented here indicates that caraway seeds are rich source of polyphenolic compounds such as phenols and flavonoids. The anti-inflammatory activities of the phenolic extract of the caraway seeds could be due to the presence of these compounds. These findings provide scientific evidence for usingthe natural products as promising source for the treatment of several inflammatory diseases.Further *in vivo*detailed studies using the caraway phenolic extract should be interesting and could lead to the development of potential sources of novel anti-inflammatory drugs.

Table 1. Proximate composition of caraway seeds on dry weight basis (g/100g)

Parameter	Amount (%)*
Moisture	4.2 ± 0.10
Carbohydrate	50.1 ± 0.96
Protein	7.6 ± 0.06
Lipid	8.7 ± 1.50
Ash	4.1 ± 0.47
Fiber	25.3 ± 1.56

*Values are mean± SEM of three estimations.

Table 2. Phenolic compounds in caraway seeds

Phenolic Compound	Concentration* (µg/g of seed powder)
Caffeic acid	475±18
Ferulic acid	350±14
Gallic Acid	148±11
Cinnamic acid	125±13
Catechuic acid	105±16
Quercetin	129±15
Kaempferol	69±12

*Values are mean± SEM of three estimations.

Table 3. HPLC retention time and fragments of phenolic compounds identified from phenolic extract of caraway through LC-MS

Retention time	$[M-H]^-$	Fragmented ion	Corresponding fragment	Compound
6.15	169	125	$M-COO^-$	Gallic acid
8.19	153	109	$M-COO^-$	Catechuic acid
13.25	179	135	$M-COO^-$	Caffeic acid
17.44	300.8	170	M-125	Cinnamic acid
		125	Trihydroxy benzene fragment	
21.02	193	178	$M-O^-$	Ferulic Acid
		149	$M-COO^-$	
29.00	301.1	151	M- Free phenol at 2 position and a portion of the benzopyranone ring moiety	Quercetin
37.09	285	133	M-151	Kaempferol
		151	Free phenol at position 2 and a portion of the benzopyranone part	

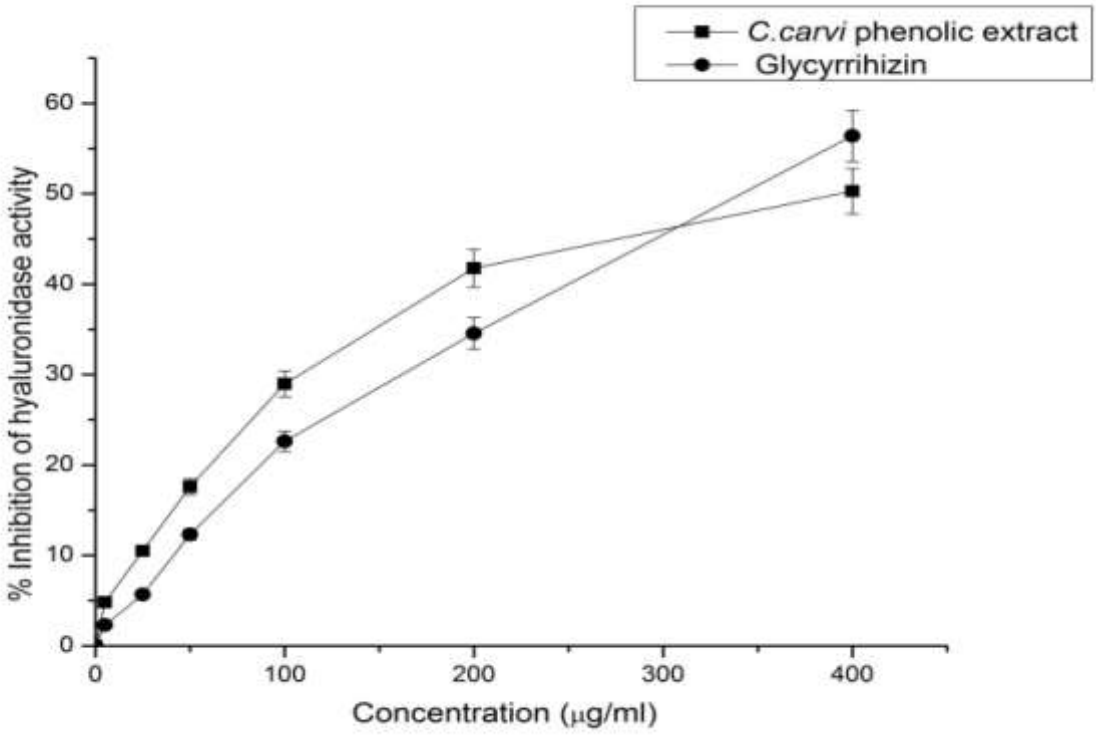

Figure 1.Dose dependent inhibition of hyaluronidase activity by caraway phenolic extract and glycyrrhizin. The values are mean ± three SEM of three independent experiments.

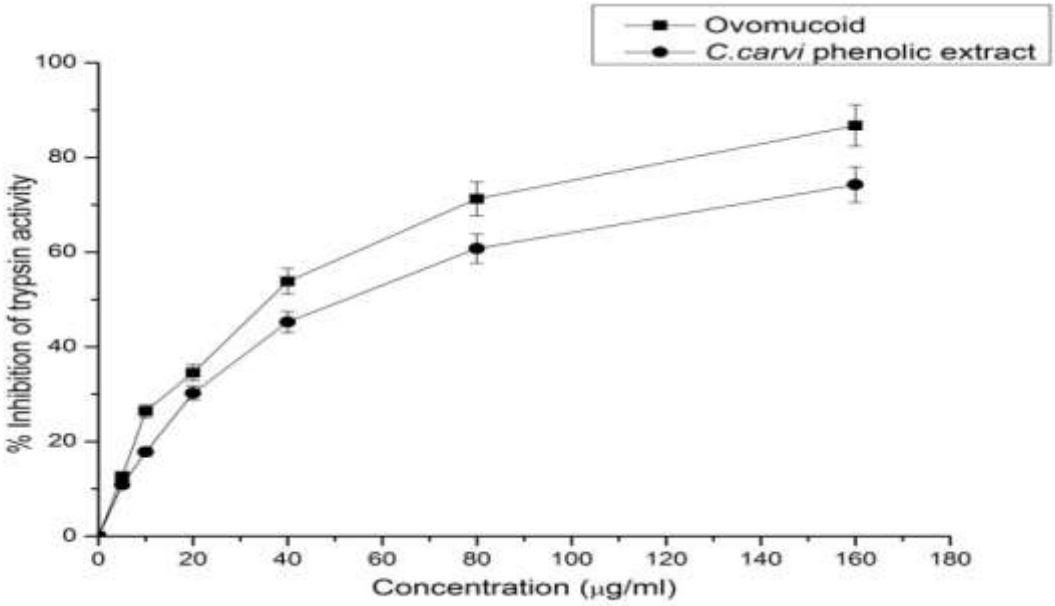

Figure 2.Dose dependent inhibition of trypsin activity by caraway phenolic extract and ovomucoid. The values are mean ± SEM of three independent experiments.

REFERENCES

1. Holtmann G et al. Effects of a fixed combination of peppermint oil and caraway oil on symptoms and quality of life in patients suffering from functional dyspepsia. Phytomedicine 2003; 10: 56-57.
2. Madisch A et al. Treatment of functional dyspepsia with a herbal preparation: a double blind, randomized, placebo-controlled, multicenter trial. Digestion 2004; 69: 45-52.
3. Coon T,Earnst EJ. Systematic review: herbal medicinal products for non-ulcer dyspepsia, Alimen. Pharmacol. Therap 2002; 16: 1689-1699.
4. Reynolds JEF. The Extra Pharmacopoeia, 30th ed. Pharmaceutical Press, London, 1993,pp. 1349–1350.
5. Bellakhdar J. La Pharmacop´ eeMarocaineTraditionnelle, M´edecineArabeAncienneSavoirsPopulaires. Edition Ibis Press, 1997, p-150.
6. Khayyal MT et al. Antiulcerogenic effect of some gastrointestinally acting plant extracts and their combination. Arzneimit-telforschung 2001; 51: 545–553.
7. Zheng G et al. Anethofuran, carvone, and limonene: potential cancer chemopreventive agents from dill weed oil and caraway oil. Planta Med 1992; 58: 338–341.
8. Nakano Y et al. Antiproliferative constituents in Umbillifereae plants II. Screen-ing for polyacetylenes in some Umbellifereae plants, and isolation of panaxynol and falcarindiol from the root ofHeracleummoellendorffii. Biology and Pharmacology Bulletin 1998; 21: 257–261.
9. Eddouks M et al. Caraway and caper: potential anti-hyperglycaemic plants in diabetic rats. J. Ethnopharmacol 2004; 94: 143–148.
10. Cotran R et al. Pathologic basis of disease. 5th ed. WB Saunders: Philadelphia, 1994. pp. 1-34.
11. Fries J. Approach to the patient with musculoskeletal disease. 19th ed. WB Saunders: Philadelphia, 1992,pp 1488-91.
12. Heras I et al. Effects of ISO 9000 certification on companies' profitability: an empirical study, *6th International Conference on ISO 9000 and TQM*, Ayr, Scotland, 2001.
13. Mayatepet E, Hoffmann GF. Leukotrienes: biosynthesis, metabolism, and pathophysiologic significance. Pediatric Research 1995; 37: 1-9.
14. Surh YJ et al. Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS trough suppression of NF-kB activation. Mutation Research 2001; 49: 243-268.
15. Bernstein PR et al. Inhibitors of human leukocyte elastase. Progress in Medicinal Chemistry, Elsevier: Amsterdam 1994; 37: 59-120.
16. Harris E. The second international anti-cardiolipin standardization workshop/the KongstonAntiphospholipid Antibody study (KAPS) group. American Journal of Clinical Pathology1990; 94 (4): 476-484.
17. Watts RA, Isaac JDL. Immunotherapy of rheumatoid arthritis. Ann Rheum Dis Sci 1992; 51: 577–580.
18. Cragg GM et al. Natural products in drug discovery and development. J Nat Prod 1997; 60 (1): 52-60.
19. Viuda-Martos M et al. Spices as functional foods- Critical Reviews in Food Science and Nutrition 2011; 51(1): 13–28.
20. Kris-Etherton PM et al. Bioactive compounds in foods: Their role in the prevention of cardiovascular disease and cancer. American Journal of Medicine 2002; 113(9): 71s–88s.
21. Singh M et al. Challenges for research on polyphenols from foods in Alzheimer's disease: bioavailability, metabolism, and cellular and molecular mechanisms. Journal of Agricultural and Food Chemistry 2008; 56 (13): 4855–4873.
22. Kaefer CM, Milner JA. The role of herbs and spices in cancer prevention. Journal of Nutritional Biochemistry 2008; 19 (6): 347–361.
23. Pan MH et al. Anti-inflammatory activity of natural dietary flavonoids. Food and Function 2010; 1(1): 15–31.
24. Hertel W et al. Inhibitory effects of triterpenes and flavonoids on the enzymatic activity of hyaluronic acid-splitting enzymes. Arch. Pharm. Chem. Life Sci 2006; 339: 313-318.
25. Prasad SN et al. Spice phenolics inhibit human PMNL 5-lipoxygenase. Prostaglandins, Leukotrienes and Essential Fatty Acids 2004; 70: 521–528.
26. Laughton MJ et al. Inhibition of mammalian 5-lipoxygenase and cyclooxygenase by flavonoids and phenolic dietary additives. Relationship to antioxidant activity and to iron ion-reducing ability. BiochemPharmacol1991; 42: 1673-81.
27. Dubois M et al. Colorimetric method of determination of sugars and related substances. Anal. Chem. 1956; 28: 350-356.
28. Slinkard K, Singleton VL. Total phenol analyses: automation and comparison with manual methods. Am. J. Enol. Viticult 1977; 28: 49-55.
29. Asada MM et al. Inhibitory effect of alginic acids on hyaluronidase and on histamine release from mast cells. BiosciBiotechnolBiochem1997; 61: 1030-32.
30. Cannell RJP et al. Results of a large scale screen of microalgae for the production of protease inhibitors. Planta Med 1988; 54(1): 10–14.
31. ThippeswamyNB et al. Antioxidant and antibacterial properties of phenolic extract from *Carumcarvi*L. J. of Pharm. Res. 2013; 7: 352-357.
32. Girish KS,Kemparaju K. A low molecular weight isoform of hyaluronidase: Purification from Indian cobra (Najanaja) venom and partial characterization.Biochemistry (Moscow) 2005; 70: 708-712.
33. Parellada J, Guinea M. Flavonoid inhibitors of trypsin and leucineaminopeptidase: A proposed mathematical model for IC_{50} estimation. Journal of Natural Product 1995; 58 (6): 823–829.