JKAU: Sci., **vol. 15,** pp. 139-150 (1423 A.H. / 2003 A.D.)

Preliminary Study on the Biological and the Biochemical Effects of Cloves Spice

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ABSTRACT. In this study, extracts of whole cloves in n-hexane (a non-polar solvent) were tested (*in vitro*) against the three pancreatic digestive enzymes, α-amylase, chymotrypsin and lipase. The results show that the extract has no significant effect on α-amylase activity ($P > 0.05$) by a mean (± SD) of 36.3 ± 4.11 μ/mg, while the chymotrypsin activity was increased significantly ($P < 0.01$) by a mean (± SD) of 3.80 ± 0.12 μ/mg, and serum lipase activity was reduce significantly ($P < 0.05$) by a mean (± SD) of 280 ± 4.5 μ/mg. The study was also included (*in vivo*) experiment to assess the influence of the spice extract on enzymatic systems such as ALP, LDH, GPT, GOT, GGT and CPK as well as their effects on some clinically important biomolecules in the serum such as glucose, cholesterol, total protein, urea and inorganic elements.

Various differences were observed in the effect of the extract on the activities of enzymatic systems and other biochemical parameters. The results obtained were compared with related work carried out elsewhere.

Introduction

Many spices and spice seeds are employed as adjuncts to impart flavor and aroma or piquancy to foods in different cultures worldwide. Among the spices consumed in Saudi Arabia is cloves (*Syzygium aromaticum*). In old medicine (traditional), cloves were used to help stimulate the heart, digestion, strengthens the mouth gums, gives a nice smell to the mouth and helps pregnancy[1]. In modern medicine, cloves can be prescribed for fever, as a detergent and analgesic. It cures the boils, headaches and fits. It helps also digestion, analgesic for toothache, improves the eczema, and activates the heart and stomach[2]. Its powder can be used for weakness of the stomach and for diarrhea and different type of vomiting[3]. Cloves contain about 14-21% of volatile oil, 10-13% of tannin, var-

ious triterpene acids and esters[4]. Cloves oil contains 84-95% of phenols (eugenol with about 3% acetyleugenol), sesquiterpenes (α, β caryopyllenes) and small quantities of furfural, vanillin and methyl aryl ketone[5]. A previous study compared the sensitivity of some human pathogenic bacteria and yeast to various spice extracts and commonly employed chemotherapeutic substances. Of the different spice tests only garlic and cloves were found to possess antimicrobial activity[5]. Cumin, onion and clove oils found significantly suppressed alfatoxin production, reduced or completely suppressed CO_2 evolution of *Aspergillus's fumigates*[6].

These results suggest that the use of spice oils may offer some advantage in the prevention of myotoxin production[6]. A study on the anti-mold activity of clove oil was made by means of micro structural observation; the result showed that clove oils have strong anti-mold activity and chemo preventive agent[7-8]. A recent study indicated that cloves are highly anti-oxidant activity[9]. Eugenol, an essential oil in cloves, significantly inhibited tobacco-induced mutaginicity at concentration of 0.5 and 1.0 mg/L, it is also inhibited the nitrosation of methylurea in a dose dependent manner[10]. The five active known compounds in the clove terpenes: β caryophyllene, β caryophyllene oxide, α-humulene, α-humulene epoxide and eugenol showed significant activity as inducers of the detoxifying enzyme glutathione S-transferase (GST) (EC 2.5.1.18) in the mouse liver and small intestine[11]. The ability of natural anti-carcinogens to induce detoxifying enzymes has been found to correlate with their activity in the inhibition of chemical carcinogenesis. The study suggested that these sesquiterpenes show some promise as potential anti-carcinogenic agents[11]. In regards to the immune system, the inhibitory effects of cloves and cardamon on the histamine production and histidine decarboxylase (EC 4.1.1.22) activity of *Morganella morganii* (a potent histamine-producing bacteria in fish) was examined at 30ºC using HPLC[12]. In the study, cloves exhibited a significant inhibitory effect. As in the *in vitro* study, cloves showed a significant inhibitory effect on histamine, putrescine and tyramine production but not on that of cadaverine. Cardamon exhibited moderate effect and pepper was ineffective. The study concluded that cloves are more helpful than cardamon in the minimization of the formation of toxic histamine[12].

Materials and Methods

Chemicals and Spice

All chemicals and solvents used in this study were of the purest analytical grade (Ultra Pure). They were purchased from different local agents for Sigma, Merck and BDH companies. Samples of the spice cloves (*Syzygium aromaticum*) were bought from local market and were used in this study.

Extraction of Sample

Dried weight of whole cloves (45-100 g) was used for extraction. The sample was soaked for 20 days in a dark bottle containing 0.5L of n-hexane. By filtering through filter paper, the residues were discarded and the crude n-hexane extracts were re-filtered with Whatman filter paper (42 ashless, Whatman Ltd., UK).

The solvents were removed by evaporation at 80ºC using model R110 Buchi Rotary Evaporator (USA). The weight of the extract was recorded and then dissolved in 10 ml acetone[13].

Animals

Adult male Wister white rats aged 3-4 weeks and weighing 250-350 g were obtained from the Animal House of King Fahad Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia. The rats were fed on the rat and mouse breeding diet cube and water, both available *ad libitum*. The initial and final body weights were recorded for each experimental animal and were divided into three main groups.

Group One: Control Rats

A total of 5 normal rats were housed for 30 days and were used as control groups, they were put under the same physiological conditions as the test animals.

Group Two: Treated with Corn Oil

A total of other 5 normal rats were housed for 30 days in separate animal cage, and were treated with corn oil only. The treatment involved a forced feeding technique of 1.0 ml corn oil fed to rats while anaesthetized by diethylether by using of a special feeding syringe[13]. This treatment was conducted for 30 days (once every two days). The aim of this group is to observe and compare any effect of corn oil to the serum parameters when mixed with cloves extract and fed rats.

Group Three

A total of 5 normal rats were housed for 30 days in a separate animal cage, and were fed with cloves extract at 733 mg/kg per day for a period of 30 consecutive days. The treatment involved a forced feeding technique of the extract dissolved in a 1.0 ml corn oil (once every day).

Collection of Blood Sample and Separation of Serum

At the end of each experiment, blood samples were collected from rats, by cardiac punctures under light ether anesthesia, into plain tubes. Blood sera were separated by centrifugation at 3000 g for 20 min. (Labo fuge. Model 2000, Co., Heraeus, USA) and stored at 0º- (–20ºC) until used for the determination of serum enzymes and other biochemical components.

Elemental Analysis

One gram from the dried sample of the cloves was placed in a small beaker, then 10 ml of analytical grade concentrated HNO_3 (6 N) was added and allowed to stand overnight. Sample was then heated carefully on hot plate, cooled and then 4 ml of 70% (v/v) of $HClO_4$ was added. The mixture was heated again to evaporate to yield a concentrated small volume. The sample was transferred to a flask and diluted to 50 ml volume with deionized water. Elemental analysis of the spice was carried out using Perkin-Elmer, model 5000 atomic absorption spectrophotometer[14]. The determination of elements including: Na, K, Ca, Mg, Al, Ba, Cu, Mn, Zn, P, Fe, Ni, Pb, Sr, Cr and Si.

Determination of Serum Enzymes and Other Biochemical Parameter

The blood sera separated from group three rats by centrifugation at 3000 g for 20 min. were divided into two parts. Part one was used to determine the serum enzymes such as Alkaline Phosphatase (ALP), Lactate Dehydrogenase (LDH), Glutamic Pyruvate Transaminase (GPT), Glutamic Oxaloacetate Transaminase (GOT), Gamma Glutamyl Transferase (GGT) and Creatine Phosphokinase (CPK). The other part was used to determine the biochemical parameters, Glucose, Cholesterol, Total Protein and Urea, using an automated system (Boehringer Mannheim Hitachi Spectrophotometer, Model 4020)[15-19], available at King Khalid National Guard Hospital, Jeddah, Saudi Arabia.

Effects of Extracts on α-amylase Activity

The assay method of α-amylase activity was based upon the hydrolysis of α-1-4 links of starch with the production of reducing sugars described by Fisher and Stein[20]. The α-amylase activity assay was carried out at pH 6.9 in isotonic medium in a total volume of 6 ml containing 1 ml potassium phosphate buffer 0.1 M at pH 6.9, 0.5 ml of sodium chloride (10 g/L), 2.5 ml of buffered starch substrate (5g/L phosphate buffer), 50 µL aliquots of cloves extract and 0.5 mL of α-amylase enzyme (5 mg/50 ml buffer 0.1M).

The volume was made up to 5 ml with distilled water. The mixture was incubated for 5 min. at 37ºC. Adding 0.5 ml of sodium hydroxide 2 M terminated the reaction. Then 0.5 ml of dinitrosalicylic reagent was added to each test tube. The mixture was heated for 5 min. in a boiling water-bath, and then tubes were cooled and read at 540 nm.

Effects of Extracts on α-chymotrypsin Activity

Delmer *et al.* described the assay method of α-chymotrypsin activity[21]. The reaction was carried out at pH 6.2. 20 µL of α-chymotrypsin pipette into a series of numbered test tubes, including a blank with 20 µL distilled water. Aliquots (10 µL) of cloves extract were added to each tube. Control experiments were run simultaneously using acetone (10 µL) alone. 1 ml of Tris buffer 0.1 mM was added to each tube and finally 5 µL of substrate succinyl-ala-ala-prophe-p-nitroaniline (10 mg/5 ml Tris buffer) were added. The mixture was incubated for 3 min. then read at 380 nm.

Effects of Cloves Extract on Lipase Activity

Activity of lipase was determined according to Sigma procedure, which is based upon the amount of fatty acids formed and originally described by Tietz and Fierech[22]. The lipase activity was carried out by adding 10 ml of Sigma lipase substrate (Olive oil 50% (v/v) and sodium azine 0.1%), 2.5 ml of distilled water and 1 ml of 0.2 M Trizma buffer, pH 8.0. Adding 1 ml of serum lipase to the test tubes except the blank tube, 50 µL of aliquots of cloves extract ran the reaction, for blank tube 50 µL of acetone were added instead. The mixture was incubated in a constant temperature water-bath at 37ºC for 3 h.

After starting the incubation, 1 ml of serum lipase was added into the blank. At the end of incubating period, 3 ml of 95% ethanol were added to each test tube and then six drops of 0.9% (w/v) thymolphthaline indicator. The test tubes were then titrated with 0.05 N of NaOH to slight but definite blue color.

Results and Discussion

1 – Extraction of Cloves

Different dried weights of (45-100 g) of cloves (whole) were used with n-hexane solvent. The highest yield of dried weight extraction obtained was 4.40 g.

2 – Elemental Analysis

Table 1 shows the analytical results of the elements content in the cloves. The data obtained showed that the clove contains more than 15 elements varies in quantity. Na^{+}, K^{+}, Ca^{+2}, Mg^{+2}, Al^{+3} and Fe^{+2} were present in large quantities

especially Ca^{+2} (2.03%) and K^{+} (1.73%). Other elements were absent, such as Zn^{+2} and Si^{+2}. Cloves in this study were found to be the richest source of Na^{+} (about 0.6%) compared to the other spices studied by other colleagues in the same department such as cardamon and areca nut (unpublished data). This finding is in full agreement with the result of Murphy *et al.*[23]. The highest quantity of elements in cloves is Ca (2%), K (1.7%) and Mg (0.9%). Cloves is the only spices that contain aluminum (Al) (0.5%) compared with other spices such as cardamon and areca nut (unpublished data), therefore cloves could be considered as a natural good source of Al such as tea and other herbs and spices[24]. Zn and Si were not detected and the other elements were found in trace amounts such as Ba, Cu, Pb, Sr and Cr.

TABLE 1. Elemental analysis of clove spice by atomic absorption spectrophotometer model 5000 Perkin-Elmer.

Elements*	Cloves (% w/w)	Element	Cloves (% w/w)
Na	0.56	Zn	Nil
K	1.73	P	0.26
Ca	2.03	Fe	0.72
Mg	0.91	Ni	0.013
Al	0.50	Pb	0.002
Ba	0.008	Sr	0.012
Cu	0.0018	CR	0.02
Mn	0.18	Si	Nil

*One gram of dried whole cloves were placed in a small beaker. 10 ml of analytical grade concentrated HNO_3 was added and allowed to stand overnight, then heated carefully on hot plate, cooled and then 4 ml of 70% $HClO_4$ was added.

3 – Extraction of Cloves Spice Used in the in vitro Enzymatic Studies, Effect of Cloves Extract on α-amylase, α-chymotrypsin and Lipase Activities

The effect of n-hexane extracts of cloves on α-amylase α-chymotrypsin and lipase activities *in vitro* are presented in Table 2. The results showed various effect of the extraction against three digestive enzymes. The amylase (EC.3.2.1.1), which is present in salivary glands and the pancreas primarily responsible for the digestion of starch to maltose, as a major end product was not significantly affected. This finding is in full agreement with the work of Reddy *et al.*[25]. Chymotrypsin (E.E.2.4.21.1), which is present in the pancreas as one of several proteases that preferentially hydolyzes peptide bonds involving carboxyl groups of aromatic amino acids[26] was significantly increased in its ac-

tivity to over 300% ($P \leq 0.01$) and by means (± SD) of 3.8 μ/mg ± 0.12. The extract at concentration 63 mg/kg increased the activity of chymotrypsin more than 3 fold. This may suggest that eugenol and other phenolic substances present in cloves oil are responsible for this increase in chymotrypsin activity, but additional proofs is certainly needed.

TABLE 2. Effect of n-hexane extract of cloves on a-amylase, a-chymotrypsin and lipase activity *in vitro*.

Enzymes	Extraction concentration (mg/kg)	Mean activity of control (μ/mg)	Mean activity of experiment (μ*/mg)	Significances	% Activity
α-amylase (± SD)	51	41 (± 0.1)	36.3 (± 4.11)	n.s.	88.53
α-chymotrypsin (± SD)	63	1.15 (± 0.02)	3.80 (± 0.12)	**	330.43
Lipase (± SD)	58	506.8 (± 4.4)	280 (± 4.5)	*	55.25

n-hexane dry extract was dissolved in 10 ml acetone and then 100 μl was added to each reaction medium to find final concentration as indicated;

Acetone 100 μl was used as control.

Results represent the average of 3 independent experiments.

n.s. = non-significant

* = Significant ($P < 0.05$)

** = Highly significant ($P < 0.01$)

* Units / mg for α – amylase calculated according to eq $= \frac{\text{micromoles maltose liberated}}{\text{mg enzyme in reaction} \times 5 \text{ min}}$

Units / mg for α – chymotrypsin calculated according to eq $= \frac{\text{micromoles P – nitroaniline}}{\text{mg for enzyme reaction} \times 3 \text{ min}}$

Units / mg for lipase adjusted to standardized curve.

The lipase (EC.3.1.1.3) which is presently mainly in the pancreas, and responsible for the hydrolysis of triglycerides long-chain fatty acid showed a significantly decrease in its activity by 45% ($P < 0.05$) and by mean (±SD) of 280 μ/mg ± 4.5. This finding is not surprising in light of previous studies showed that the essential component of cloves oil is eugenol produce inhibitory effect on pancreas lipase[12].

4 – Effect of Cloves Extract on some Biochemical Parameters of Rats' Serum in vivo

The effects of n-hexane extract on some biochemical components of rat serum such as (Glucose, Cholesterol, Total protein and Urea) are shown in Table 3. Cloves n-hexane extraction increased the concentration of glucose about

(165%) ($P < 0.01$) by mean (± SD) of 20.40 nmol/L ± 0.72. The results indicate that cloves extract produced a significant hyperglycemic effect and potentiated insulin activity more than three-fold which is very similar to results obtained by Khan *et al.*[27]. They had tested several foods and spices for their potential action of insulin, among the spices, apple pie spice, cinnamon, cloves, bay leave and turmeric. The cloves extract showed no significant effect on cholesterol value ($P > 0.05$) by mean (± SD) of 1.28 mmol/L ± 0.07 although other spices caused significant reduction of serum cholesterol[28]. The total plasma protein was examined in this study and showed no significant increase ($P > 0.05$) by mean (± SD) of 58.76 g/L ± 0.08. The cloves extract showed a slight decrease in urea concentration by mean (± SD) of 3.7 ± 0.07. Unfortunately, there is no more data available in literature concerning these two parameters to be compared with our findings.

TABLE 3. Effect of n-hexane of cloves spice (dissolved in corn oil) on some biochemical components of rat serum.

Sample	Glucose mmol/L	Cholesterol mmol/L	Total protein g/L	Urea mmol/L
Control (± SD)	7.70 (± 1.39)	1.54 (± 0.05)	53.67 (± 0.06)	5.5 (± 0.1)
Cloves (± SD)	20.40** (± 0.72)	1.28 (n.s.) (± 0.07)	58.76 (n.s.) (± 0.08)	3.7* (± 0.07)

n.s. = non-significant
* = Significant ($P < 0.05$)
** = Highly significant ($P < 0.01$)
The results represent the average of 3 independent experiments.

5 – Effects of Spices Extract on some Key Enzymes of Rat Serum in vivo

Table 4 shows the effect of n-hexane extract of cloves on six serum enzymes: Alkaline Phosphatase (ALP), Lactic Dehydrogenase (LDH), Glutamic Pyruvic Transaminase (GPT), Glutamic Oxaloacetic Transaminase (GOT), Gamma Glutamyl Transferase (GGT) and Creatine Phosphokinase (CPK). The results exhibited a significant decrease in the activities of ALP, LDH, GPT, GOT and CPK ranged between 35% such as ALP, GPT and CPK ($P < 0.05$) and over 50% such as LDH and GOT ($P < 0.01$) whereas GGT produced a highly significant increase activity (110%), ($P < 0.01$). The results are comparable to the effect of other spices such as garlic, ginger and a combination of garlic plus ginger on serum biochemical parameters[29]. However, there is no more data study available on effect of cloves extract on the above serum enzymes studied. Spices have been used in food preparation and for medicinal purposes throughout

the world, but still the question remains why people of nearly all over the world use spices? But no single answer, yet several hypothesis are made as well as hundreds of spices still need more investigation.

TABLE 4. Effect of n-hexane extracts of the cloves on some rat serum enzymes *in vivo*.

Sample	ALP (μ/ml)	LDH (μ/ml)	GPT (μ/ml)	GOT (μ/ml)	GGT (μ/ml)	CPK (μ/ml)
Control (± SD)	138.3 ± 0.18	1173.3 ± 4.4	62.00 ± 0.8	195.3 ± 3.3	10.67 ± 0.7	1049.3 ± 3.32
Cloves extract (±SD)	93.88* ± 8.9	603.0* ± 0.7	40.0* ± 2.6	79.7* ± 4.7	21.67** ± 1.04	654* ± 8.8

ALP : Alkaline Phosphates.
LDH : Lactic Dehydrogenase.
GPT : Glutamic Pyruvic Transaminase.
GOT : Glutamic Oxaloacetic Transaminase.
GGT : Gamma Glutamyl Transferase.
CPK : Creatine Phosphokinase.
*Significant = ($P < 0.05$)
**Highly significant = ($P < 0.01$)
The results represent the mean of triplicate independent experiment.

Acknowledgment

Many thanks to my colleague Dr. Ahmed N. Abo-Khatwa for continuous support, my thanks also conducted to my graduate student M.S. Ba-Rjjash.

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دراسة مبدئية للتأثيرات الحيوية والبيوكيميائية لتابل القرنفل

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جـــدة – المملكة العربية السعودية

المستخلص. تعتبر البهارات من أهم المحسنات للطعم والمذاق للأغذية كما تستخدم كعلاج شعبي في بعض الحالات المرضية طبقًا للمادة الفعالة التي تحتويها . وفي هذه الدراسة تم استخدام مذيب الهكسان الأقل قطبية لاستخلاص المادة الفعالة في بذرة القرنفل وقد وجد أنها تحتوي على مادة اليوجينول كأحد المكونات الرئيسة بالإضافة إلى مكونات أخرى عديدة منها مادة أستيل يوجينول . وقد تم دراسة تأثير هذا المستخلص على ثلاثة إنزيمات هضم داخلية وهي ألفا أميليز ، وألفا كيموتربسين وإنزيم الليبيز في أنابيب الاختيار (in vitro). ودلت النتائج أن نشاطية إنزيم ألفا أميليز لا تتأثر بقيمة معنوية في حين وجد أن نشاطية إنزيم الألفا كيموتربسين تزداد بنسبة ٣٠٠٪ ، أما إنزيم الليبيز والمسؤولة عن تكسير ثلاثي الجليسريدات فإن نشاطاتها تقل بنسبة ٤٥٪ . كما تم دراسة تأثير هذا المستخلص على بعض الأنظمة الإنزيمية داخل الخلية (in viro) مثل إنزيم الفسفوتيز القاعدي ، وإنزيم اللاكتيت دي هيدروجينيز ، وإنزيم الجلوتامك بيروفيت ترانس أمينيز وإنزيم الجلوتامك أوكسالو اسيتايت ترانس أمينيز وإنزيم الجاما جلوتاميل ترانس فريز وإنزيم الكرباتين فسفوكاينيز . ودلت النتائج أن مستخلص بذر القرنفل تقلل من نشاطية كلٍ من إنزيم الفسفوتيز القاعدي وإنزيم الجلوتامك بيروفيت تراس أمَينيز وإنزيم الكرياتين فسفوكاينيز بنسبة تتراوح ما بين (٣٠-٤٠٪) في حين إنزيم اللاكتيت دي هيدروجينز وإنزيم الجلوتامك أوكسالو أسيتايت ترانس أمينيز بنسبة أكثر من ٥٠٪ . أما إنزيم الجاما جلوتاميل ترانس فريز فإن نشاطية الإنزيم تزداد بنسبة (١١٠٪). كذلك تم دراسة تأثير هذا المستخلص على بعض المكونات الحيوية مثل تركيز الجلوكوز ، الكوليسترول والبروتين الكلي واليوريا ودلت النتائج أن تركيز الجلوكوز تزداد بنسبة ١٦٥٪ ، في حين

نجد أن الكوليسترول والبروتين الكلي لم يحدث فيها تغيير ذو قيمة معنوية . أما تركيز اليوريا فانه يحدث فيه انخفاض نسبي بسيط (٣٠٪) . وقد دلت النتائج التي تحصلنا عليها أن هناك بعض الاختلافات وبعض التشابهات عند مقارنتها مع الدراسات الأخرى في نفس المجال .