

Biochemical Effects Of A Methanolic Extract Of Cloves On Rats And Some Enzymes

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Abstract: The effects of the methanol extract of the spice clove (*Syzygium aromaticum*) on the three digestive enzymes; α -Amylase, chymotrypsin and lipase were studied (*in vitro*). The findings of results showed that the extract has no significant effect on α -amylase activity 44.0 ± 0.0 U/mg (mean \pm SD) with P-value of > 0.05 , increased chymotrypsin activity significantly 1.78 ± 0.12 U/mg (mean \pm SD) with P-value of < 0.01 , and reduced serum lipase activity slightly 486 ± 7.1 U/mg (mean \pm SD) with P-value of $. 0.05$. The study also assessed the influence of the extract on enzymatic levels ALP, LDH, GPT, GOT, GGT, and CPK (*in vivo*). The results show that the activities of LDH, GPT, and GOT were increased by 154%, 302%, and 348% respectively, while the activities of ALP, GGT, and CPK were decreased by 55.2%, 37.5% and 58.6% respectively. The study also examined the effect of extract on serum levels of Glucose, Cholesterol, Total protein, Urea and Inorganic Elements. The results are discussed in reference to related literature work and the impact of extraction with polar versus non-polar solvents.

Key words: Clove, Spice, digestive enzymes, methanol, extract.

Introduction

The spice clove (*Syzygium aromaticum*) is very commonly consumed in Arabian and South-East Asian countries because of its piquancy. In old medicine, cloves were used to help digestion, stimulate the heart, prevent vomiting in pregnancy and strengthen the mouth gums. In new medicine, cloves can be prescribed for fever as analgesic and other purposes. Eugenol, the major component of clove oil has significant medicinal properties. It significantly inhibits tobacco-induced mutagenicity at concentrations of 0.5-1.0 mg/L (Sukumaran and kuttan. 1995). In the immune system, clove has inhibitory effect on histamine production and histidine decarboxylase (Shakila, *et al.* 1996). Clove oil

inhibits also the production of superoxide anions (O_2^-) from macrophages *in vitro* (Joe and Lokesh. 1994). The components of Cloves oil contain 84-95% of phenols (about 3% acetyl eugenol), sesquiterpenes (α -humulene, α -humulene epoxide, β -caryophyllene, β -caryophyllene oxide and eugenol), and small quantities of furfural, vanilline and methyl arylketone. (Zhang, *et al.* 1992., Merk index. 1989). Eugenol and acetyl eugenol inhibited arachidonate, adrenaline and collagen-induced platelet aggregation and these components were antiaggregatory by a combination of at least two effects; (i) inhibition of platelet thromboxane formation and (ii) increased formation of 12-lipoxygenase products (Saeed and Gilani, 1994). It was reported that both eugenol and acetyl eugenol are more potent

than aspirin in inhibiting platelet aggregation induced by arachidonate, adrenaline and collagen (Srivastava 1993). (Hassan and Mahmoud 1993) reported that cloves oil significantly suppressed aflatoxin production. Other studies examined the effect of eugenol oil on severe chronic adjuvant arthritis in rats; the study showed a significant suppression of paw and joint swelling. This finding suggests that eugenol have potent anti-inflammatory and/or antirheumatic properties (Sharma, *et al.* 1994). Some studies using eugenol indicated an ameliorating effect on environmental mutagens, especially those present in food (Soudamini, *et al.* 1995). As antimicrobial activity, (Arora and Kaur, 1999) as antioxidant compound that provide significant protection against chronic disease (Craig, 1999) and as dental analgesic (Myint, *et al.* 1996). The present study was carried out to extract the active ingredients from clove seeds by using of a polar solvent methanol to study the effect of this extract on some biochemical parameters in vivo and in vitro.

Materials and Methods

Chemicals and spice

All chemicals used in this study were fine grade chemicals, and were purchased from different sources through local distributors. The spice cloves (*Syzygium aromaticum*) were bought from local markets. Enzymes used in the in vitro tests, α -amylase (bacterial source), α -chymotrypsin (from bovine pancrease), and serum substrate of human lipase were purchased from Sigma Chemical Co. (St. Louis USA), Lipase obtained from human serum.

Animals

Adult male Wister rats, (average weight

250-350 g) were randomly selected from a rat colony bred at the experimental animal unit of King Fahad Medical Research center. Animals were fed standard diet produced by Grain, Silos and Flour Mills Organization, Western province, Saudi Arabia. The animals were divided into three groups, and the initial and final weight was determined for each group.

Group I: A total of 5 rats for each experiment were housed for 30 days and served as control for group II. They were treated under standard conditions.

Group II: A total of other 5 rats for each experiment were housed for 30 days in separate animal cage, and were treated with corn oil only. The treatment involved forced feeding technique of 1.0 ml corn oil while anesthized by diethyl ether by using of a special feeding syring (Abo-Khatwa & Kubo, 1987). This treatment was conducted for 30 days (once every two days). The aim of this group was to observe and compare any effect corn oil may have on the serum parameters when mixed with cloves extract and fed rats.

Group III: A total of other 5 rats for each experiment were housed for 30 days and were fed with methanolic cloves extract at 635 mg/kg/day for a period of 30 consecutive days. The treatment involved a force-feeding technique of the extract dissolved in 1.0 ml corn oil.

Extraction of sample

Whole dried cloves (55-100 g) were soaked for 20 days in a dark bottle containing 300 ml of 95% methanol. The residues were removed by filtering through filter paper and were discarded. The crude methanol extract was filtered twice using Whatman filter paper (No. 42 ashless). The solvent was evaporated at

Table 1. Comparison of Dry weight extract of cloves by Methanol and n-hexane.

Solvent	Dry weight of cloves (g)	Dry weight of extract (g)	%
Methanol	50.5	4.81	9.50
n-hexane	50.40	2.30	5.50

90°C using a rotary evaporator, and the weight of the extract were recorded before dissolving in 10 ml acetone.

Collection of blood sample and separation of serum

At the end of 30 days feeding rats with clove extracts, blood samples were collected by cardiac punctures (under light ether anesthesia) into plain tubes. Blood sera were separated by centrifugation at 3000 rpm for 20 min and stored at (-20°C)-0°C until determining of serum enzymes and other biochemical parameters.

Elemental analysis

Elemental analysis of cloves was carried out by placing one gram of dried sample (whole cloves) in a small beaker, and then 10 ml of concentrated HNO₃ (6N) was added and allowed to stand over night. The sample was then heated carefully on a hot plate, cooled, and then 4 ml of 70% (V/V) HClO₄ was added. The mixture was heated again to concentrate the sample, which was then transferred into a flask and diluted to 50 ml with deionized water. Elemental analysis was carried out using Perkin-Elmer model 5000 atomic absorption spectrophotometer (Isaac and Johnson, 1975).

Determination of α -amylase activity in vitro

The assay of α -amylase activity was based upon the method described by Fischer

and Stein (1961). The assay was carried at pH 6.9 in isotonic medium in a total volume of 6 ml containing 1.0 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 (5 g/L phosphate buffer), 50 μ L aliquots of cloves extract and 0.5 ml of α -amylase enzyme (5 mg/50 ml buffer 0.1 M). The volume was made up to 5 ml with distilled water. The mixture was incubated for 5 min. at 37°C. Adding 0.5 ml of sodium hydroxide 2.0 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.

Determination of α -Chymotrypsin activity in vitro

The Delmer, *et al.* (1979) method was used to determine the effect of cloves extract on α -chymotrypsin enzyme activity at pH 6.2. A 20 μ L volume of α -chymotrypsin (5 mg/5 ml Tris buffer 0.1 mM) was added to a series of numbered test tubes including a blank with 20 μ L distilled water. Aliquots (10 μ L) of cloves extract were added to each tube, except the control tube which (10 μ L) of acetone was added. Then 1 ml of Tris buffer 0.1 mM was added to each tube, and finally 5 μ L of substrate succinyl-ala-ala-pro-phe-p-nitroaniline (10 mg/5 ml Tris buffer) were added. The mixture was incubated for 3 min. at 25°C. The

reaction complete releasing of p-nitroalanine in approximately 5 min and read at 380 nm.

Effect of cloves extract on lipase activity

Activity of lipase was determined according to Sigma procedure, which was based upon the amount of fatty acids formed. Originally described by Tietz and Fierech (1966). The assay was carried out by adding 10 ml of Sigma lipase substrate (Olive oil 50% (V/V) and sodium azide 0.1%), 2.5 ml of distilled water and 1 ml of 0.2 M Trizma buffer, pH 8.0. 1 ml of human serum lipase was added to the test tubes except the blank tube, 50 μ L of aliquots of cloves extract ran the reaction, for the blank tube 50 μ L of acetone were added instead. The mixture was incubated in constant temperature water-bath at 37 °C for 3h.

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

Determination of serum enzymes & other biochemical

The blood sera collected from group III

rats by centrifugation were divided into two parts. Part one were used to study the effect of clove extract on some 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 second part was used to determine the biochemical parameters, such as Glucose, Cholesterol, Total protein and Urea. The measurement was carried out by using an automated system (Boehringer Mannheim Hitachi spectrophotometer, Model 4020).

Results and Discussion

Extraction of Cloves

The use of methanol to extract the active principles of cloves gave high yield of extract (9.5% based on dry weight). The initial 50.5 g dry weight of cloves gave 4.81 g of dry extract (9.5%). This yield was nearly twice that obtained with n-hexane (Table 1) clove extract contains eugenol, which is the chief phenolic substance. (Myint, *et al.* 1996).

Elemental Analysis

Results presented in Table 2, show elements of content in cloves. The study revealed that the clove contains more than 15

Table 2. Elemental Analysis of methanolic extract of clove's spice.

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

* The elemental analysis carried out by using Perkin-Elmer, 5000 atomic absorption spectrophotometer.

Table 3. Effect of the Methanolic extract on three digestive enzymes in vitro.

Enzymes	Extraction concentration mg/kg	Mean activity of control U/mg \pm SD	Mean Activity of experiment U/mg \pm SD	Significant	% activity
α -amylase	102	41.0 (\pm 0.15)	44.0 (\pm 0.0)	n.s	107.56
Chymotrypsin	120	1.150 (\pm 0.02)	1.783 (\pm 0.12)	.	155.04
Lipase	120	506.8 (\pm 4.4)	486.4 (\pm 7.1)	n.s	95.97

Methanol dry extract was dissolved in 10 ml acetone and 50 μ l was added to each reaction medium to give final concentrations as shown in table.

n.s = non-significant $P > 0.05$

+ = 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 of α -chymotrypsin calculated according to eq. = $\frac{\text{Micromoles P-ntroaniline}}{\text{mg for enzyme in reaction} \times 5 \text{ min.}}$

Units/mg for lipase adjusted to standardized curve.

The mean represent triplicate independent experiments.

elements varies in the 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 or present in trace quantities such as Zn^{+2} and Si^{+2} . Cloves are the only spices containing aluminum (AL^{+3} 0.50%) compared to other spices such as cardamom and areca nut (unpublished data). Therefore, cloves could be considered as a natural source of Al such as tea and other herbs (Pennington, 1988).

Effect of clove extraction on α -amylase, α -chymotrypsin and serum lipase activities in the (in vitro)

Table 3; showed the effect of the clove extract on the activities of three digestive enzymes of great importance. The α -amylase (EC. 3.2.1.1) is responsible for the digestion of starch to maltose as a major end product. The clove extract had no significant effect on the activity of the enzyme at 102-mg/kg concentrations. Chymotrypsin (EC. 2.4.21.1) is one of the pancreatic proteases that preferentially hydrolyzes peptide bonds of aromatic amino acid residues (Calbreath,

1992). The methanolic extract of cloves at 120 mg/kg concentration increased the activity of enzyme by 155% [$P < 0.01$]; mean (\pm SD) of 1.78 U/mg \pm 0.12]. The lipase (EC. 3.1.1.3), responsible for the hydrolysis of triglycerides was not significantly affected by the clove extract. The latter result contradicted a previous study by the same author (Khan, 2001), where n-hexane was used for extraction. Previous studies showed that the essential component of cloves inhibited pancreatic lipase (Rona and Pavlovic, 1922., Murray and King, 1929).

Effect of Methanolic extract of cloves on some Biochemical components in vivo

Table 4, shows results concerning the effect of methanolic extract of cloves on levels of Glucose, Cholesterol, total protein and Urea in rat serum.

There was a slight increase in glucose concentration (36.7%) but no hyperglycemic effect. This is consistent with a previous study (Khan, *et al.* 1990), which showed that cloves potentiated insulin activity. The "cloves factor(s)" that potentiated the action of insulin

Table 4. Effect of methanolic extract of cloves on some biochemical components in vivo.

Sample	Glucose (mmol/l)	Cholesterol (mmol/l)	Total protein (g/l)	Urea (mmol/l)
Control (mean) (± SD)	7.70 ± 1.39	1.54 ± 0.06	53.67 ± 0.06	5.5 ± 0.1
Cloves (mean) (± SD)	10.53* ± 2.66	1.48* ± 0.13	61.00** ± 0.56	6.2* ± 0.1

* Non-significant $P > 0.05$ ** Significant $P < 0.05$

The mean represent triplicate independent experiments.

in glucose metabolism is still unknown (Khan, *et al.* 1990). The methanolic extract of cloves had no significantly effect on cholesterol levels. Other spices are known to have strong hypolipidemic effects. For example, ethanolic extracts of nutmeg produced significant lower values of lipids, (total cholesterol, TGL

of rat serum was unaffected by methanolic extract.

Effect of Cloves Extract on some key Enzymes of rat serum in vivo

The results presented in Table 5 showed the effect of methanolic extracts of cloves on

Table 5. Effect of Methanolic extract of cloves spice on some rat serum enzymes in vivo:

Sample	ALP (U/ml)	LDH (U/ml)	GPT (U/ml)	GOT (U/ml)	GGT (U/ml)	CPK (U/ml)
Control (mean) (± SD)	138.33 ± 0.18	1173.33 ± 4.4	62.0 ± 7.8	195.33 ± 3.32	10.67 ± 3.32	1049.33 ± 3.32
Cloves (mean) (± SD)	76.33** ± 2.52	1801.33** ± 1.54	187.0** ± 1.02	680.0** ± 1.01	4.00* ± 1.00	614.76** ± 0.88

* = Significant $P < 0.05$ ** = Highly significant $P < 0.01$

The mean represent the values of 3 independent experiments.

(triglycerides) and LDL (low-density lipoprotein) (Ram, *et al.* 1996), also curry leaves and mustard seeds reduced total serum cholesterol and other lipids such as LDL and VLDL (Khan, *et al.* 1995). The total plasma protein of rat serum increased with methanolic extract of clove by nearly 10-15%. No data are available in the literature regarding the effect of spices on plasma protein. The Urea content

some key enzymes of rat serum. It is clear that clove extract caused significant increases in LDH, GPT, and GOT, and decreased ALP and GGT and CPK. The activity of alkaline phosphate was reduced markedly (55.2%). Lactate dehydrogenase increased to nearly 154%. The extract also increased the activity of GPT and GOT to 302 and 348%, respectively. Both enzymes are known to increase markedly

in a variety of liver disorders. The extract of cloves also reduced the activity of creatine phosphokinase (CPK) by nearly 58.6%, but had a slight effect on gamma glutamyl transferase (GGT). It has been estimated that approximately 40 different spices are used in our diet today (Henry and Emery, 1986). These spices contain a wide variety of active phytochemicals, including terpenoids, flavanoids, lignans, polyphenolics and other components and still a lot of studies and investigations are needed on these natural products.

In conclusion, the results presented in this study have shown that the use of polar solvent (methanol) to extract the active principles of clove gave high yield compared with non-polar solvent (n-hexane). In addition, present of mineral salts in this extract. The impact of the extract against three important digestive enzyme (in vitro) have shown different effects, no significant effect on α -amylase activity which is clinically known that increasing α -amylase activity in human serum is indicator to acute and chronic inflammation, viral infection (such as hepatitis), liver diseases and cancer (Calbreath, 1992). Significantly increased α -chymotrypsin activity, which may due to present eugenol and other phenolic components, and slightly decreased lipase activity, which can be explained to the inhibitory effects of clove oil on histidine decarboxylase, which regulates the histamin production (Shakila, *et al.* 1996). The results of the study have shown also variety effects of extract on some biochemical parameters and key enzymes activities in serum (in vivo). However, spices have long been implicated as a cause of gastric mucosal injury. Yet, by the use of endoscopy, it was concluded that ingestion of highly spiced meals by normal individuals was not associated with any stomach damage (Graham, *et al.* 1988).

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التأثير الكيموحيوي لمستخلص القرنفل بواسطة الميثانول على الفئران وبعض الانزيمات

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المملكة العربية السعودية

الملخص العربي: تم دراسة تأثير مستخلص توابل القرنفل (*Syzygium aromaticum*) بواسطة الميثانول على ثلاثة أنواع من انزيمات الهضم وهي ألفا أميليز، ألفا كيموتربسين والليباز في أنابيب الاختبار، وقد أظهرت النتائج أن مستخلص القرنفل ليس له تأثير معنوي على انزيم الألفا أميليز 0.00 ± 0.44 وحدة/ملجرام ($P > 0.05$)، وازداد نشاطية انزيم الكيموتربسين بقيمة معنوية 78.1 ± 1.2 وحدة/ملجرام ($P < 0.01$)، في حين تناقص نشاطية انزيم الليباز بدرجة طفيفة 0.486 ± 1.7 وحدة/ملجرام ($P > 0.05$). كما اشتملت الدراسة على تأثير المستخلص على مستوى الانزيمات CPK, GGT, GOT, GPT, LDH, ALP داخل الخلية. وأظهرت النتائج أن نشاطية كلا من GOT, GPT, LDH زادت بنسبة 154% و 302% و 348% على الترتيب، في حين أن نشاطية انزيمات CPK, GGT, ALP قد تناقصت بنسبة 2.55% و 5.37% و 6.58% على الترتيب. كما تم دراسة تأثير المستخلص في الرشاحة على مستوى الجلوكوز والكوليسترول والبروتين الكلي واليوريا والعناصر غير العضوية، وقد تم مناقشة النتائج على ضوء النتائج في الدراسات المشابهة وتأثير المستخلص بواسطة مذيب قطبي مقارنة بمذيب غير قطبي.