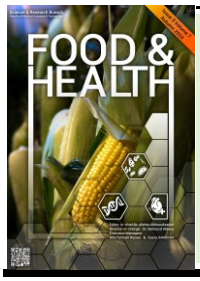

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Investigating the antimicrobial properties of thymol coated with xanthan and guar on the proliferation of Staphylococcus aureus and Listeria in hamburger

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ABSTRACT

This study investigated the impact of thymol and thymol coated with xanthan gum and guar on the antibacterial properties of hamburgers. The study also examined the effects on acidity and the total volatile nitrogen ratio of the hamburgers over a 21-day period. After preparing raw hamburgers made with beef, fried and a total of eight treatments were examined, including burgers without thymol, those with 0.5% thymol, and those with 1% thymol. Additionally, variations included burgers with 0.5% thymol coated with 1% xanthan gum, 0.5% thymol coated with 0.5% guar gum, 1% thymol coated with 1% xanthan gum, 1% thymol coated with 1% guar gum, and 1% thymol coated with 0.5% xanthan and 0.5% guar gum. The results of the comparative analysis of the average acidity of the samples containing thymol and coated with xanthan and guar showed that the acidity index in the samples containing thymol coating with xanthan and guar was significantly lower than the control treatment. The lowest level of TVN was observed in the TCXG treatment while the highest level was observed in the control treatment. In addition, the total volatile nitrogen index showed a significant increase over time. The most effective treatment in controlling the population of yeast, staphylococcus aureus and listeria compared to the control treatment was the treatment of 1% thymol coated with 1% xanthan and guar. So that the populations of yeasts 0.97 log CFU/g, Staphylococcus aureus 1.17 log CFU/g and Listeria 0.52 log CFU/g were reduced compared to the control treatment.

1. Introduction

Due to the process of globalization and the active trading of food items, foodborne illnesses instigated by bacteria, fungi, viruses, and parasites have emerged as a prominent global health concern (1, 2). Global spread of pathogens through international Trade is due to the mass export of animals and their meat (Kouba, 2015). Consumption of contaminated food products results in approximately 600 million cases of infection and 420,000 deaths per year (3). Microorganisms linked to risks associated with food safety predominantly result in self-restricting ailments characterized by symptoms such as nausea, vomiting, abdominal pain, diarrhea, and headaches (1, 4). In general, meat is among perishable foods. Meat is distinguished by its vulnerability to spoilage by microorganisms and oxidation (5). Raw meat frequently harbors various bacteria like Salmonella, Campylobacter, and Escherichia coli O157:H7 (6).

Hamburger is one of the protein products that are prepared from minced red meat of beef or veal or a mixture of both with mutton. The meat is made into rounds, then fried or cooked on charcoal. It has different burgers and its sandwich with lettuce, mushrooms, pickles, tomatoes and round bread is known as one of the most popular foods in the world. Neglecting to cook hamburgers properly increases the likelihood of pathogenic bacteria in the food at the point of consumption, which can have serious implications for public health (7).

To reduce of contamination by food-borne pathogens, the food industry has implemented advanced techniques for

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processing and storage (8).On the other hand, with the increasing tendency of consumers towards food processed with natural additives, the preference for using natural ingredients over synthetic compounds has increased. For example, among the natural additives that have received attention today, we can refer to essential oils (EOs) and plant extracts or the compounds obtained from them (9).

Although the utilization of medicinal and aromatic plants (MAPs) along with their EOs in culinary practices dates back to ancient civilizations (9), their significance in the realm of biopreservation has only recently garnered considerable attention. Within this category of plants, thyme (Thymus vulgaris L.) emerges as a particularly promising reservoir of bioactive compounds, such as EO and thymol, which exhibit the ability to inhibit the proliferation and dissemination of undesirable microorganisms. Nevertheless, the precise antimicrobial mechanisms of these substances at the molecular and cellular levels remain incompletely elucidated, resulting in a limited availability of commercially manufactured EO-based food preservatives in the present day (10, 11).

The incorporation of naturally derived antimicrobials (e.g., plants, EOs, and other extracts) into food represents an effective strategy for the control of spoilage, the inhibition of the proliferation of pathogenic microorganisms (12), and the enhancement of consumer acceptance. EOs derived from MAPs have emerged as optimal alternatives to synthetic food additives, exhibiting a robust antimicrobial effect and reduced health concerns (13, 14).

It has been demonstrated that important compounds such as thymol can readily penetrate the cell membrane of microorganisms, thereby disrupting them. This results in the disruption of the enzyme system, loss of cell content and, ultimately, cell death (15). Nevertheless, essential oil compounds may have disparate targets, and their antimicrobial effect is dependent on a multitude of mechanisms of action, which affect respiration, energy metabolism, genetic material, cell wall, or cell membrane (16). It is challenging to ascertain the susceptibility of specific strains. As a result, it is challenging to ascertain the susceptibility of particular strains (16). Furthermore, Gram-positive bacteria demonstrate greater susceptibility to cell wall-targeted EOs than Gram-negative species, which possess an outer membrane (15,17). The presence of a hydrophilic lipopolysaccharide membrane restricts the traversal of hydrophobic compounds, thereby rendering Gram-negative bacteria more tolerant to EO compounds (12). In contrast, the cell wall of Gram-positive bacteria is more complex. It contains 90-95% peptidoglycan, which allows for the diffusion of hydrophobic compounds (18).

Conversely, the gums present in meat products facilitate the preservation of moisture, a reduction in cooking loss, an improvement in cutability properties, a greater degree of juiciness, a more favourable mouthfeel, and an enhancement of the product's overall colour. It is possible to cite the examples of xanthan and guar gum. The combination of two or more gums has been shown to yield favourable results in meat products. The reaction of xanthan gum with guar gum has a positive synergistic effect.

In this study, the effect of thymol and thymol coated with xanthan and guar on the growth of bacteria and related traits such as volatile nitrogen compounds and acidity have been investigated.

2. Materials and methods

2.1. Extraction of thymol aqueous extract

This study employed the MICROSYNTH model microwave oven, manufactured in Italy and capable of delivering 700 W of power. To extract aqueous extract, the balloon containing the sample (*Zataria multiflora*) and water (50 gr and 750 ml of distilled water) is placed inside the microwave chamber, with the clunger installed in the upper part of the microwave. The microwave was set to a power level of 700 w and operated for a period of 45 minutes. Given that the density of the extract is less than that of water, the extract formed a distinct phase in the upper part of the apparatus. The heavier water was placed in the lower portion and subsequently returned to the flask containing the sample and water through the return mechanism. This process was repeated. The extraction process was continued until the level of the extract in the collection vessel remained constant for 45 minutes. After, one microlitre volume of each extracted extract was injected into the GC/MS machine for analysis of the compounds present.

2.2. Microencapsulation

The microencapsulation process entailed the combination of thymol (at concentrations of 0.5% and 1%) with xanthan and guar. Dimethyl sulfoxide (DMSO) solvent was used to prepare thymol concentrations. Initially, 0.01 gr of thymol was dissolved in 1 ml of DMSO. Following that, 1 ml of Tween 80 emulsifier (for uniformity and homogeneity) was added to the solution. Then 100 ml of xanthan and guar were added separately under controlled temperature conditions and the mixture was homogenized at 4000 rpm using a homogenizer for 5 minutes in each step (for a total of 10 minutes). To preserve the structural integrity of the microcapsules and the thymol within, the resulting solution was transferred to a plate and subjected to freeze-drying (to remove solvents). This same methodology was employed in the preparation of microcapsules containing 1% guar and a combination of guar and xanthan (0.5% guar and 0.5% xanthan, respectively).

2.3. Making hamburgers

The daily slaughtered beef was purchased and used from the Tehran market, then 80% lean beef and 20% fat, was subjected to a double rotation process utilising a meat grinder. Subsequently, minced meat, comprising 60% by weight, was combined with other ingredients of 40% by weight, including onion (28%), vegetable oil (4%), breadcrumbs (3%), and dry fat-free. It should be noted; that these percentages refer to the composition of the hamburger mixture excluding the encapsulated antioxidant. The preparation of hamburgers involved the use of milk (3%), salt (1%), spices (0.5%), sodium polyphosphate (0.3%) and spice water (0.2%). Before

forming the hamburger, 400 mg/kg of encapsulated gallic acid was added to the mixture for every 100 grams of beef.

Afterward, the hamburger was shaped using a steel mould with a weight of 100±5 gr, a thickness of 0.5 cm and a diameter of 9 cm. In the next step, in order to prevent moisture loss, the hamburger was enclosed in a polyvinyl chloride (PVC) film and kept at a freezing temperature of -18 °C. Following that, the encapsulation efficiency was employed to ascertain the quantity of capsules comprising 400 ppm of gallic acid. The remaining steps were conducted in a manner identical to that of the control sample, which lacked the encapsulated antioxidant. Control and treated hamburgers were subjected to analysis at 0, 15, and 30-day intervals throughout the storage period at -18 °C (Table 1).

Table 1. Different treatments in this study.

Row	Treatments	Description
1	CON	burger, without thymol
2	TYM0.5	burger containing 0.5 % of thymol
3	TYM1	burger with 1% of thymol
4	TCX0.5	burger with 0.5% of thymol coated with 1% xanthan gum
5	TCG0.5	burger with 0.5% of thymol coated with 1% guar gum
6	TCX1	burger with 1% of thymol coated with 1% xanthan gum
7	TCG1	burger with 1% of thymol coated with 1% guar gum
8	TCXG	burger with 1% of thymol coated with 1% xanthan and guar gum

2.4. Determination of encapsulation efficiency

The quantity of extract entrapped within microcapsules was determined through the application of the method proposed by Niu et al. (2020), with certain adaptations. To ascertain the encapsulation efficiency, the initial 1 ml of each sample was subjected to centrifugation at 1300 rpm for 30 minutes, thereby facilitating the separation of the capsules. Next, to completely remove unseparated particles, the samples were filtered using a 0.22-micron syringe head filter. 40 µl of each sample was taken and diluted to 2 ml using methanol. The absorbance was then read by a UV-visible spectrophotometer model 4802 (Jenway, UK) at a wavelength of 275 nm, in accordance with the methodology described by (19).

2.5. Measurement of volatile nitrogen substances (TVB-N)

For this purpose, 10 gr of the sample were combined with 2 gr of magnesium oxide and 300 ml of distilled water, with the addition of a few stones and a few glass pearls (to prevent foaming). The flask was heated for 15 minutes until the boiling point was reached. The vapours from the distillation flask were collected directly in the Erlenmeyer flask containing 25ml of a 2% boric acid solution and a few drops of methyl red reagent until the total volume of boric acid and condensed vapours inside the Erlenmeyer flask reached 150 ml. eventually, the solution resulting from the accumulation of distillation vapours was titrated with 0.1 normal sulfuric acid until the colour reached that of onion skin. The quantity of nitrogen material present in the sample was determined in mg (20).

2.6. pH measurement

A 10% sample of meat paste was prepared and homogenised with water for the purpose of measuring the pH of the sample using a digital pH meter model PL-700PV (EZDO, Taiwan) (21).

2.7. Counting bacteria

In a sterile environment, the sample container was opened and 10 gr of the sample was separated using sterile forceps and scissors. Then it was placed in a special sterile plastic bag and filled with 90 ml of sterile Ringer's solution. The bag was homogenised by transferring it to the Stomaker (Bagmixer, Interscience, France) for 3 minutes. Subsequently, 1 ml was added to the tube containing 9 ml of Ringer, resulting in the preparation of the subsequent dilutions. After preparing serial dilutions, these were then cultured on plates containing plate count agar (PCA) culture medium (Merck, Germany) using the surface culture method. To enumerate the aerobic mesophilic bacteria, the cultured plates were incubated at 37 °C for 48 h. To enumerate the psychrotrophic (cold-tolerant) bacteria, the plates were incubated at 7 °C for 7 to 10 days. on the other hand, 100 microliters of samples were cultured in Baird Parker Agar and palkam selective Agar (Merck, Germany) for enumeration viable cells of staphylococcus aureus and listeria and the plates incubated at 37 °C for 48 h (22, 23, 24).

2.8. Statistical Analysis

By performing various tests on samples, the data were recorded in Excel software. SPSS version 21 software was used for statistical analysis of the data, and P value less than 0.05 was considered statistically significant. Analysis of variance test was used to check the significant difference between treatment and control conditions. Duncan's statistical test was used to compare the difference between means at the 0.05 level. If the distribution of the data obtained from the research is normal, repeated measure ANOVA was used to investigate the changes in physicochemical, microbial and sensory characteristics in the treatments.

3. Results

3.1. Acidity and total volatile nitrogen index

The results of the comparative analysis of the average acidity of hamburger samples containing thymol and coated with xanthan and guar revealed that the coating of thymol with xanthan and guar resulted in a notable reduction in acidity indices in comparison to the control treatment (Table 2). Therefore, the control treatment exhibited the highest level of

acidity, while the treatment involving thymol coated with xanthan and guar demonstrated the lowest acidity. From the commencement of the trial (day 1) to day 21, there was a notable increase in the acidity index.

The investigation of total volatile nitrogen indices in fried hamburgers demonstrated that the utilisation of thymol and coated thymol resulted in a considerable reduction in the TVN index. Thus, the lowest amount of TVN was observed in TCXG and TCX1 treatments with 10.99 and 10.99 mg/100 g, respectively, and the highest amount was observed in the control (15.29 mg/100 g). Additionally, the total volatile nitrogen index exhibited a significant increase over time ($P< 0.05$).

Table 2. Effect of Thymol Coating with Xanthan and Guar on pH and Total Volatile Nitrogen in Fried Hamburgers.

Treatment	pH	Total volatile nitrogen
CON	6.05±0.02^{a}	15.29±0.11^{a}
TYM0.5	5.96±0.04^{b}	13.59±0.13^{b}
TYM1	5.81±0.01^{c}	11.84±0.05^{d}
TCX0.5	5.79±0.03^{c}	11.76±0.01^{e}
TCG0.5	5.81±0.02^{c}	11.94±0.04^{c}
TCX1	5.74±0.01^{e}	11.35±0.05^{g}
TCG1	5.77±0.00^{d}	11.55±0.10^{f}
TCXG	5.72±0.00^{f}	10.99±0.07^{h}

Different lowercase letters indicate significant differences in the treatments ($P< 0.05$).

3.2. Microbial population

Table 3. The impact of thymol and thymol-coated with xanthan and guar on the microbial population during the storage period (Mean±sd).

Treatment	Yeast	*S. aureus*	Listeria
CON	4.05±0.06^{a}	4.53±0.07^{a}	7.05±0.13^{a}
TYM0.5	3.76±0.05^{b}	4.08±0.11^{b}	6.08±0.06^{b}
TYM1	3.31±0.01^{e}	3.72±0.05^{c}	5.82±0.07^{c}
TCX0.5	3.57±0.06^{c}	3.62±0.04^{d}	5.79±0.05^{c}
TCG0.5	3.55±0.04^{c}	3.75±0.06^{c}	5.81±0.08^{c}
TCX1	3.32±0.03^{e}	3.54±0.03^{e}	5.75±0.11^{c}
TCG1	3.39±0.02^{d}	3.64±0.01^{d}	5.77±0.13^{c}
TCXG	3.08±0.06^{f}	3.36±0.09^{f}	5.53±0.07^{d}

Different lowercase letters indicate significant differences in the treatments ($P< 0.05$).

As shown in Table 3, the population of yeast, Staphylococcus aureus and Listeria in the hamburger samples under test exhibited a notable increase with the passage of time, particularly with the prolongation of storage. Additionally, the utilisation of thymol and thymol coated with xanthan and guar resulted in a notable reduction in the population of yeast, Staphylococcus aureus and Listeria in hamburger samples when compared to the control treatment. According to the results, the count of yeast, Staphylococcus aureus and Listeria in TCXG samples with 3.08, 3.36 and 3.53 log CFU/g respectively was significantly lower than other samples and it was more effective in controlling the growth of these microbes, while these values were reported in control samples 4.05, 4.53 and 7.05 log CFU/g respectively.

4. Discussion

4.1. Volatile total nitrogen index

Regarding the TVN values, a decline was observed during the storage periods in comparison to the control. This is attributed to the efficacy of thymol as a natural antioxidant in beef. This finding aligns with the observations of Andres et al. (2014) and is in compliance with the stipulations of Standard No. 2688 of 1987, which specifies that TVN values should not exceed 14 mg/100 g of meat (25).

TVNs are amino compounds that are produced during the spoilage of high-protein food products, particularly meat. The microbial activities occurring in meat during the storage period exert a significant influence on the production of TVNs. The results of the TVN analysis of different burger samples during storage time are presented in the Table 2. The quantity of TVN in all samples increased over time, and a statistically significant discrepancy was observed, which is associated with the bacterial catabolism of amino acids, resulting in the accumulation of ammonia, various ethylamines, and volatile bases. This result is consistent with the findings reported by Hemtian et al. (2022), who monitored the freshness of rainbow trout fillets in a gelatin film containing Coleus scutellarioides. Their findings indicated an increase in TVN rate over 16 h at 25 °C (26). A reduced quantity of TVN was evident in burgers comprising thymol, coated with xanthan and nanoencapsulated guar. This finding aligns with the observations made by Hosseini et al. (2016), who reported a rise in TVN levels in all fish fillets during the storage period. The study demonstrated that fish fillets coated with gelatin and oregano essential oil exhibited a reduction in TVN during the storage period (27). The number of bacteria increased over time, resulting in elevated TVN production. The bioactive compounds in CEO disrupt the bacterial outer membrane and release lipopolysaccharides, which indirectly reduce TVN. A previous study also reported that the use of plant extracts could reduce the TVN value of meat products due to the preservative effects of phenolic compounds (28).

4.2. pH of hamburger sample

The present study revealed a notable elevation in pH levels during storage ($P<0.05$). The pH values of the various burgers exhibited an upward trajectory, with all samples displaying a correlation with the volatile base compounds generated by microbial and endogenous enzymes. Some samples exhibited an acceptable pH value of less than 6.5, which was attributed to the gradual release of phenols during storage. The application of coatings to individual samples had a significant impact on the pH values observed. Notably, the samples coated with thymol and xanthan/guar exhibited a lower pH than the

control. These findings align with those of other studies which have demonstrated that the pH value of meat products increases over time. The possible mechanism stated is that, microbial enzymes cause the release of amino acids by hydrolyzing enzymes and proteins, and because amino acids have buffering properties, they neutralize the lactic acid in the meat tissue and ultimately lead to an increase in the pH of the samples (28, 29, 30).

4.3. Bacterial population

Meat and its products are highly perishable and, in the absence of appropriate storage conditions, are susceptible to spoilage. Once these products have spoiled, they become unfit for human consumption due to microbial growth and chemical changes brought about by enzymes. In general, lipid oxidation is considered to be one of the most significant factors contributing to the deterioration of meat quality. It is responsible for the development of an unpleasant taste and odour, an increase in drip loss, a loss of pigmentation, and ultimately, a reduction in consumer acceptability (22). This deterioration is primarily attributable to contamination of meat and meat products during the production, handling, and consumption processes. In addition to cross-contamination during processing, poor personal hygiene and improper storage temperatures also contribute to microbial growth, which can cause spoilage and contribute to foodborne illness in humans, resulting in serious health problems. The most significant pathogenic bacteria associated with meat products are: Salmonella spp., Staphylococcus aureus, Listeria monocytogenes, Clostridium perfringens, Clostridium botulinum, Escherichia coli O157:H7, Pseudomonas, Acinetobacter, Enterobacter, Lactobacillus sp. and Proteus (22, 31).

It is anticipated that the proliferation of bacteria in meat can be curtailed or entirely prevented through the preservation of meat products via chilling and freezing (32). Alternative techniques, such as the addition of sodium nitrite and potassium, have been observed to contribute to the contamination of prepared foods.

Given that meat is a rich source of protein, the preparation of high-nitrite products, including sodium nitrate and potassium nitrate, can enhance the durability of meat and poultry products. Sodium nitrite has been shown to preserve the distinctive colours, textures and flavours of meat and poultry, a quality that cannot be replicated by any other additive. However, the use of such chemical compounds has been linked to adverse effects on human health (33).

The necessity for meat preservation methods arose from the need to transport meat over long distances without spoilage and loss of nutritional value (34). EOs and their extracts can be employed as natural additives to reduce the reliance on chemical preservatives and their associated risks. These substances have the potential to extend the shelf life of meat and its products, as well as to regulate and inhibit microbial growth (35). A number of EOs have been identified as having beneficial effects in food production, due to their antibacterial, antifungal and antioxidant properties (35).

EOs are a rich source of natural antioxidants, including phenolics, flavonoids, alkaloids, tannins, and phenolic acids .The concentration of TBA is frequently employed as an indicator of lipid oxidation in meat products (36). EOs possess a variety of compounds with antibacterial properties, including geraniol, menthol, cinnamyl alcohol, linalool, citronellol, carvacrol, cinnamaldehyde, eugenol, thymol, estragole, caran and chavicol (37). The antimicrobial effects of essential oils derived from various plants have been demonstrated to effectively reduce the number of bacteria present in meat products during storage. These include coriander oil, ginger and basil oil (38), rosemary, sage and thyme oil, grape seed extract and pine bark extract, and garlic and lemongrass oils (39).

Other plants and seeds, such as cumin (Cuminum cyminum), are notable for their richness in natural compounds, including ascominaldehyde, limonene and linalool. These compounds exhibit both antimicrobial and antioxidant activity, as evidenced by their efficacy in inhibiting the growth of a range of bacterial pathogens, including Escherichia coli, Salmonella, Pseudomonas aeruginosa, Staphylococcus aureus and Listeria monocytogenes (40).

Thyme (Thymus Vulgaris) is also a good source of phenolic compounds such as g-terpinene, 1, 8-cineole, thymol and eugenol, which have antimicrobial effects on pathogenic bacteria (41). Thyme EOs successfully inhibited the growth of E. coli, Salmonella, Pseudomonas aeruginosa and Staphylococcus aureus (20).Thyme oil also has excellent antioxidant activity when added to minced meat during storage, and its high value reduces pH, TVN and TBA (39).

The present study demonstrated that the use of thymol, thymol coated with xanthan, thymol coated with guar, and thymol coated with xanthan and guar significantly reduced the population of yeast, Staphylococcus aureus, and Listeria in hamburger samples. These findings indicate that this compound has a pronounced effect on microbial population reduction. The findings of the present study align with those of previous research conducted by (28, 30).

Regarding the mechanism of action of thymol, it can be said, the antimicrobial activity of thymol is caused by changing the lipid layer of the cytoplasmic membrane and interacting with the bacterial genomic DNA. In fact, it changes the bacterial cell membrane and as a result causes the uncontrolled release of intracellular substances such as potassium ions, which are essential for the normal metabolism and survival of the bacteria (28, 30).

The addition and coating of thymol essential oil resulted in an increase in antioxidant activity and a decrease in lipid oxidation compared to the control treatment (without essential oil). These effects were observed up to 21 days after screening. Additionally, thymol was observed to affect the colour value of meat, causing it to decrease over time. This resulted in a more attractive appearance for a longer period of time. Hamburgers coated with a mixture of xanthan and guar thymol exhibited reduced cooking loss compared to other treatments.

This effect was more pronounced in the final screening periods (days 14 and 21).

Furthermore, the burgers treated with xanthan and guar were observed to exhibit greater sensitivity than the control treatment. From day 14 onwards, there was a gradual decline in tenderness, suggesting that the meat became tenderer with prolonged storage. Furthermore, the sensory characteristics of the burgers were enhanced by the coating process. This study demonstrated that thymol can extend the shelf life of processed foods, making it a potential natural alternative to artificial ingredients. The xanthan gum and guar thymol coating mixture is a suitable substitute for synthetic additives such as BHT. In addition to enhancing sensory properties, it has a favourable impact on shelf life when used in low concentrations. This was evaluated by consumers.

In conclusion, it can be stated that thymol can impede microbial and chemical alterations in beef. Additionally, the findings indicated that the thymol coating with xanthan and guar had the most pronounced impact compared to the other treatment and control samples. It demonstrated the ability to inhibit bacterial growth and, apart from the colour, enhance the chemical characteristics and sensory quality of the meat, except for the colour factor. The present study demonstrates the efficacy of thymol and its nanoencapsulated coating as a potent antibacterial and antioxidant agent with the potential to preserve and extend the shelf life of meat products. Further investigation is required to assess the impact of thymol on other meat products. Additionally, the use of packaging or other forms of coverage is recommended for the long-term storage of this novel product.

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