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Antioxidant, Antibacterial and Color Analysis of Garlic Fermented in Kombucha and Red Grape Vinegar

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Abstract

Background and Objective: Garlic, in different types, is a very common food ingredient all over the world. Traditionally, garlic is fermented in grape vinegar to produce garlic pickles; in this study, to produce a novel fermented food, garlic was fermented in kombucha beverage; then, antibacterial and chemical properties and color changes of garlics fermented in kombucha and vinegar were compared with each other and those of fresh garlic.

Material and Methods: Folin-Ciocalteu assay was performed to evaluate total phenolic contents; free radical scavenging activity was evaluated using 2,2-diphenyl-1-picrylhydrazyl. Disk diffusion method was performed to measure inhibitory activity against testing bacteria. A digital method was designed for color analysis. All data were statistically analyzed by ANOVA test at significant level of ($p \leq 0.05$).

Results and Conclusion: Fresh garlic extract had the highest inhibitory effect (mean 27.7 mm) against tested bacteria; kombucha fermented garlic showed bigger inhibition zone (mean 21.7 mm) than vinegar fermented garlic (mean 17.9 mm). Anti-*staphylococcus aureus* activity of fresh garlic was stronger than gentamycin and amoxicillin; inhibitory effect of garlic extracts against tested bacteria was significant in comparison with standard antibiotics. Fresh-garlic extract contained highest amount of phenolic contents; fermentation of garlic in kombucha decreased phenolic content of garlic bulbs by 1.92% and IC_{50} factor for antioxidant activity was 10.25% higher than fresh garlic; fermentation in vinegar reduced 21% of phenolic contents and IC_{50} obtained 47.4% higher than fresh garlic. Fermentation of garlic reduces the density of colors and luminosity, but the reduction in garlics fermented in vinegar is more than in kombucha. Appearance of vinegar fermented garlic changed to yellowish and kombucha inclined the color to reddish. Fermentation of garlic in kombucha provides better preservation of biological properties of garlic than in grape vinegar.

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1. Introduction

Garlic (*Allium sativum*), for centuries has been known as an important food ingredient and due to its preventive and curative activities, considered as a medicine. In many cultures garlic has been used as spice, flavoring and remedies [1,2]. Main biological properties of garlic are attributed to its organosulfur compounds [3,4]. Generally, garlic is consumed either raw or in processed form. Processed garlic products are mainly dried garlic, boiled, steamed, black garlic, garlic powder, tablets or capsules, fermented garlic products and etc. There are several methods to prepare different fermented products from garlic. A very popular one is called AGE; this product is prepared by soaking garlics in water/ethanol solution

and storage at room temperature for more than 10 months [2]. However garlic is commonly used as an ingredient in pickles, but there is a very popular garlic pickle which is prepared by aging garlic in vinegar for several years [5]. This pickle is popularly known as "seven years old garlic" in Iran. Traditionally it was prepared by soaking garlic under red wine or grape vinegar following by seven years of storage. Fermentation of garlic in vinegar changes its color to brown; as long as fermentation lasts, brown color changes darker. Seven years old garlic has a dark brown color and the odor of fresh garlic is almost gone.

On the other hand, kombucha is a traditional fermented beverage originated from East and has a history of several thousand years but still is popular in the West. Typical kombucha is a sweetened black tea fermented with a culture known as "tea fungus", at room temperature for about two weeks [6]. This culture (Tea fungus) is popularly called "kombucha fungi" in Iran.

Kombucha culture is actually a symbiosis of acetic acid bacteria and yeasts. The main acetic acid bacteria found in this fungus were: *Acetobacter (A.) xylinum, A. xylinoides, Bacterium* (*B.*) *gluconicum, A. aceti* and *A. pasteurianus;* and identified yeasts are: *Schizosaccharomyces* (*S.*) *pombe, S. ludwigii, Kloeckera (K.) apiculata, Saccharomyces* (*S.*) *cerevisiae, Zygosaccharomyces* (*Z.*) *bailii, Torulaspora* (*T.*) *delbrueckii, Brettanomyces* (*B.*) *bruxellensis, B. lambicus, B. custersii, Candida* (*C.*) *stellate* [7]. Potential effects like weight loss, cancer and AIDS cure have increased the interests in kombucha consumption [8,9].

Regular kombucha tea consumption contributes to weight gain inhibition and life elongation [10]. After our previous research on kombucha analogues [11], in current study kombucha was used as fermenting liquid for preparing garlic pickles. Garlic was fermented in both kombucha and red grape vinegar; then, antibacterial and antioxidant activity and color changes of fermented and fresh garlics were evaluated and compared.

2. Material and Methods

Chemical compounds: deionized water (CID: 962) bought from Zolal Teb Shimi Co. Gorgan cityIran, DPPH (CID:74358) , gallic acid (CID:370), ascorbic acid (CID:54670067) and sodium carbonate (CID:10340) all were from Merck pro-ducts bought from local chemical market in Gorgan city, Iran.

2.1. Preparation of kombucha

10 g of dry black tea (bought from a local market in Gorgan city- Iran) with 20 g sucrose were mixed in 1 L of 5 min boiled distilled water and steeped for 15 min. The mixture was cooled to room temperature and then leaves were separated. The resulting filtrate is black tea infusion which was poured into glass jar; then, the preparation was inoculated with 10 gl^{-1} of actively growing kombucha culture from our previous work [11]; and 50 ml of previously fermented kombucha was added to medium to stimulate the fermentation process. The inoculated jar lid was covered with a piece of cotton cloths and the body of container was covered with newspapers; then stored at room temperature. After 21 days, fermented liquid was passed through sterile cotton. The prepared kombucha was used as fermenting liquid for further processing [7-11].

2.2. Preparation of fermented garlics

White garlic was bought from a local market in Gorgan city, Iran. 150 g of cloves were weighted and moved into 500 ml sterile glass jars. 300 ml of kombucha was added to jar and lid was closed. Another sample was prepared by adding red grape vinegar (Taksa industry, Mashhad-Iran). The containers were covered with newspapers; jars were stored at room temperature for one month.

2.3. Extracts preparation

To prepare extracts, garlic bulbs were grated using stainless steel grater and grinded garlics were gone under pressure; exuded liquid was then passed through 0.22 micron sterile syringes filter (Biofil China) and considered as total extract. This procedure was applied for fresh garlic, kombucha fermented and vinegar fermented garlics. But for fermented garlics, bulbs were rinsed with sterile deionized water for one minute and dried with oil-free filter papers to washout fermenting liquid from bulbs surface before grinding.

Fermenting liquids (vinegar and kombucha) before and after fermentation of garlic, were also passed through 0.22 micron sterile syringes filter and were used in further evaluations. Extracts were prepared just before use [11,12].

2.4. Evaluation of antibacterial effect

Disk diffusion method, as described by The European Committee on Antimicrobial Susceptibility Testing, was followed for evaluation of antibacterial effects of extracts and fermenting liquids. [13] 0.5 McFarland solution of selected strains of bacteria (*Escherichia coli* PTCC 1395, *Salmonella typhimurium* PTCC 1596, *Staphylococcus aureus* PTCC 1436, *Staphylococcus saprophyticus* PTCC 1440, *Staphylococcus epidermidis* PTCC 1114, *Bacillus stearothermophilus* PTCC 1359 and *Pseudomonas aeroginosa* PTCC 1430) were prepared in Mueller Hinton broth. Using a sterile cotton swab, the Mueller Hinton broth cultures were swabbed on the surface of sterile Mueller hinton agar plates. 10 µl of each extract was inoculated on each 6 mm disk; when disks were dried after 3 h in 37°C incubator, they were applied on cultured plates, upside down. Inhibition zones were measured and reported in mm after 18 h

incubation at 37ºC. Gentamycin and amoxicillin discs were used as standard. All the samples were tested in triplicate.

2.5. Total phenolic content assay

2.5 ml of 10-fold diluted Folin-Ciocalteu reagent, 2 ml of a 7.5% solution of sodium car-bonate, and 0.5 ml of phenolics solution were mixed well. The absorbance was measured at 765 nm after a 15 min heating at 45°C; a mixture of deionized water and reagents was used as a blank. The content of phenolics is expressed as gallic acid equivalents [14].

2.6. Free radical scavenging activity

The electron donating ability of samples and standards gallic acid and Vit C were determined from bleaching of purple colored ethanol solution of DPPH. This spectrophotometric assay uses the stable radical 2, 2-diphenyl-1-picrylhydrazyl as a reagent. DPPH was prepared at a concentration of 2% w v^{-1}. Different concentrations of extracts were taken in separate test tubes and volumes were made up to 2 ml using distilled water. Then 2 ml of DPPH solution was added in each test tube and these solutions were kept in dark for thirty minutes. The same procedure was followed for ascorbic acid as well. All the samples were tested in triplicate. Later optical density was recorded at 517 nm using spectrophotometer (Jenway 6310-UK). Distilled water with DPPH (Merck–USA) was used as blank.

Inhibition of DPPH activity% = (A-B/A) ×100
Where A= optical density of blank; B=optical density of sample.

IC_{50} factor of samples was also evaluated; IC_{50} factor indicates the concentration or amount of extract that can inhibit 50% of free radical agent. Samples with smaller needed amount to inhibit 50% of DPPH has higher radical scavenging activity [14].

2.7. pH

pH was measured using electronic pH-meter (Denver, model 215).

2.8. Evaluation of color density

A digital, handy method was determined for color analysis. To compare color density of fresh and fermented garlics, same size cuttings of bulbs were digitally photographed (Canon A1000, 10 M pixel) at similar conditions under natural light in different ISO sensitivity (100, 200, 400, 800, and 1600).

Concentration of red, blue and green colors and luminosity of 10000 pixels from center of each sample were measured in RGB color system using adobe Photoshop CC 2014. Color with higher mean value has higher density and where standard deviation of a color is smaller, lower range of the color is reflected from surface, therefore the color has a sharper effect on appearance [15].

2.7. Statistical analysis

ANOVA test followed by Duncan multiple range test were performed at the significance level of 0.05 using SPSS software ver. 20 to statistically analyze obtained data (n=3). Microsoft Excel 2013 was used to draw chart.

3. Results and Discussion

3.1. pH and acidity of fermenting liquids

Final pH of prepared kombucha was 2.52. 8.9 ml of NaOH 0.1N was needed to neutralize 10 ml of kombucha beverage. Grape vinegar had a pH = 2.53 and 10.6 ml of NaOH 0.1N was needed to neutralize 10 ml of vinegar 10% solution.

3.2. Antibacterial effects

Mean of triplicate replication of disc diffusion test are presented in Table 1. Sadeghian & Ghazvini [16] Gull et al., [17] and Adetunde et al., [18] reported garlic juice to have strongest antibacterial activity between other types of garlic extracts, which their reports supports our finding. While fresh garlic extract showed the largest inhibition zone (mean 27.7 mm), extracts from fermented garlics had lower antibacterial effects. Kombucha fermented garlic showed bigger inhibition zone (mean 21.7 mm) in comparison to vinegar ferm-ented garlic (mean 17.9 mm). The only exception was about *P. aeroginosa* that extracts from garlics fermented in vinegar had the largest inhibitory zone between extracts. There seems to be a synergistic effect between vinegar and garlic for antibacterial activity against *P. aeroginosa*. Also it seems *S. epidermidis* is indifferent to fresh or fermented extracts of garlic and showed similar sensitivity to all garlic extracts (37 mm). The reason of this reaction is unclear and need more investigations. It can also be concluded that garlic extracts (fermented and fresh) are more effective against Gram-positive bacteria than Gram-negative. Delaha and Garagusi reported inhibitory effect of garlic against both gram positive and negative bacteria [19] and Iwalokun et al. also reported higher sensitivity of gram positive bacteria to garlic extract than gram negatives. They mentioned the lipid composition of cell walls influence on entering of active chemicals of garlic into cells [20].

About fermenting liquids, only fresh vinegar had a low antibacterial activity on *S. saprophyticus* and a significant inhibitory zone on *P. aeroginosa*. While vinegar had no effect on *P. aeroginosa* after fermentation in the presence of garlic. The reason of high inhibitory activity of vinegar fermented garlic extract against *P. aeroginosa* is more due to garlic, but vinegar played a synergistic role and increased the effect of this extract in comparison to two

others. No antibacterial activity was observed from other fermenting liquids. While anti-staph activity of fresh garlic is stronger than gentamycin (30 µg) and amoxicillin (30 µg), inhibitory effect of garlic extracts against tested bacteria is considerable in comparison with standard antibiotics used in this experiment.

Table 1. Inhibitory zone (mm) of different extracts disks applied on tested bacteria (n=3).

	Gram-positive				Gram-negative		
	S. saprophyticus	*S. aureus*	*S. epidermidis*	*B. stearothermophilus*	*S. typhimurium*	*E. coli*	*P. aeroginosa*
A	36^{aA}	39.5^{aA}	37^{aA}	33^{bA}	22^{cA}	19.5^{cA}	11.5^{dA}
B	29^{aB}	32^{aB}	37^{bA}	28.5^{aB}	20^{cA}	13^{dB}	11^{dA}
C	23^{aC}	23^{aC}	37^{bA}	19^{cC}	9^{dB}	10^{dC}	14^{eB}
D	-	-	-	-	-	-	-
E	-	-	-	-	-	-	-
F	7^{aD}	-	-	-	-	-	13^{bA}
G	-	-	-	-	-	-	-
H	27^{aB}	33^{bB}	34^{bB}	32^{bA}	25^{aC}	33^{bD}	-
I	30^{aB}	27^{bD}	31^{aC}	24^{cD}	15^{dD}	17^{eE}	17^{e}

A: fresh garlic extract; B: kombucha fermented garlic; C: vinegar fermented garlic; D: fresh kombucha; E: kombucha after fermentation with garlic; F: fresh vinegar; G: vinegar after fermentation with garlic; H: amoxicillin; I: gentamycin. Data with similar lowercase letters in each row have no significant difference ($p>0.05$). Data with similar uppercase letters in each column have no significant difference ($p\geq0.05$).

3.3. Antioxidant properties

Total phenolic contents of all samples as gallic acid equivalent are presented in figure 1. While extract from fresh garlic contains highest amount of phenolic contents, fermentation of garlic in kombucha decreased phenolic content of garlic bulbs by 1.92% and fermentation in vinegar reduced 21% of phenolic contents of garlic. Therefore it can be concluded that fermentation of garlic in kombucha provides better preservation of phenolics of garlic. Hur et al. [21] suggested that decomposition of phenolics may be due to microbial enzymes and acids produced during fermentation; also in our previous study fermentation of black tea infusion by kombucha decreased approximately 50% of phenollics; although here in current study fermentation decreased phenolics of garlic but regarding the differences in phenolic content of final products, it seems decomposition of phenolics is more due to acidity of fermenting liquid.

On the other hand, phenolic content of fermented liquids were higher than fresh liquids. It can be concluded that phenolics from garlics were extracted to liquid medium. But reduced amount of phenolics of garlic was not equal to increased amount of phenolics of fermenting liquid.

Increase in phenolic content of fermented vinegar in comparison with fresh vinegar is approx.-imately half of decrease in phenolic content of vinegar fermented garlic compared to fresh garlic. While phenolic content of kombucha doubled after fermentation with garlic and increased amount is much higher (12 times) than decreased amount of phenolic content of fermented garlic; so it can be concluded that significant part of phenolics in kombucha after fermentation with garlic is from chemical or biochemical reactions happened during fermentation process. Couto et al. [22] and Bossi et al. [23] reported that some bacteria like *A. aceti*, lactic acid bacteria and human colonic bacteria can produce some phenols; so the increase in phenolic content of fermented liquids in comparison to fresh liquids may be due to protease activity of *A. aceti* involving in fermentation process.

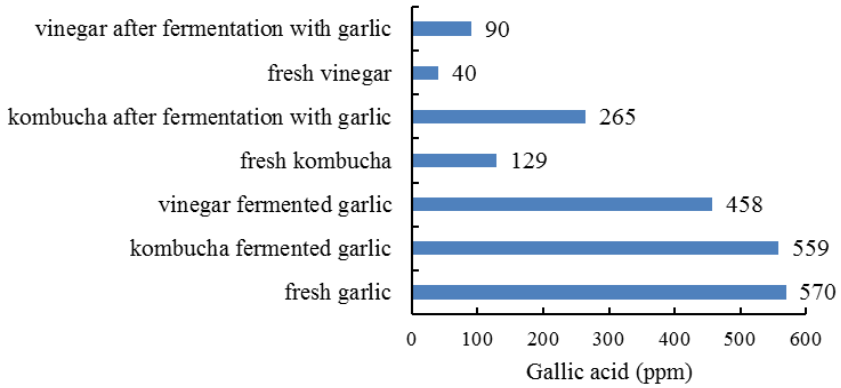

Figure 1. Total phenolic contents of garlics and fermenting liquids as gallic acid equivalent.

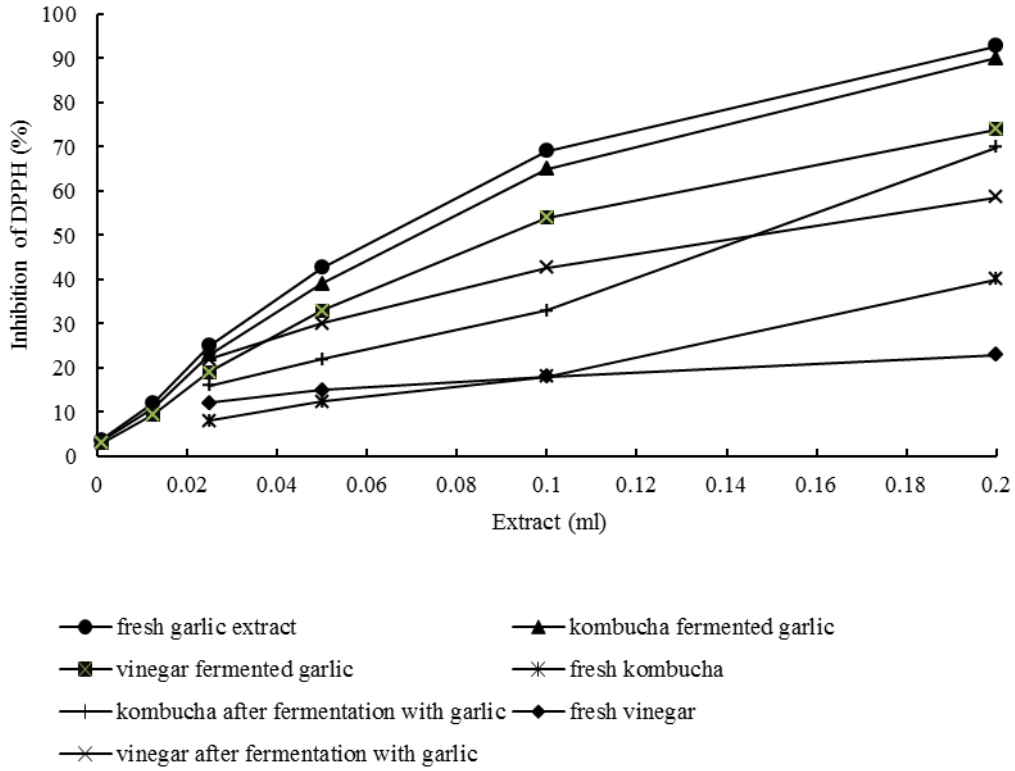

Figure 2. DPPH inhibition potential of different concentration of garlic extracts and fermenting liquids.

Figure 2 presents percent of DPPH inhibited by different concentration of each sample's extract. Comparison of IC_{50} value of samples indicates that IC_{50} of kombucha fermented garlic extract (0.086 ml) is 10.26% higher than of fresh garlic extract (0.078 ml). This value for vinegar fermented garlic obtained 0.115 ml which is 47.4% higher than fresh garlic. About the fermenting liquids, IC_{50} value of kombucha after fermentation with garlic was 0.14 ml which is 46.15% lower than fresh kombucha (0.26 ml); also vinegar after fermentation with garlic had an IC_{50} value of 0.15 ml which is 76.56% lower than of fresh vinegar (0.64 ml). 2 ml of 21.47 ppm ascorbic acid solution could inhibit DPPH by 50%.

Table 2. Digital analysis of garlic samples color (in 10000 pixels).

		Fresh garlic			Kombucha fermented garlic			Vinegar fermented garlic		
		Mean	Std. Dev	Median	Mean	Std. Dev	Median	Mean	Std. Dev	Median
ISO 100	R	136.25	6.77	136	129.44	5.01	129	92.21	7.21	92
	G	139.80	6.46	140	106.13	6.18	106	92.74	6.40	93
	B	140.89	7.32	105	60.87	9.67	61	40.04	6.20	47
	L	134.79	6.28	135	108.02	5.63	108	87.44	6.16	87
ISO 200	R	136.29	7.01	136	128.65	5.48	128	90.49	7.02	90
	G	139.61	6.55	140	106.02	6.45	106	91.24	5.95	91
	B	105.64	8.08	105	62.12	10.91	63	48.53	7.32	49
	L	134.77	6.26	135	107.86	5.78	108	86.19	5.65	86
ISO 400	R	134.05	6.80	134	126.65	5.93	127	90.07	6.62	90
	G	139.53	6.72	139	105.87	6.23	106	93.92	6.74	94
	B	106.98	5.73	107	67.69	8.15	65	52.93	5.23	53
	L	134.16	6.52	134	107.46	6.11	107	88.14	6.26	88
ISO 800	R	135.15	7.43	135	124.24	7.03	124	85.21	8.70	86
	G	140.14	7.58	140	105.43	7.28	106	91.66	7.78	92
	B	110.55	6.42	110	68.01	10.13	69	50.90	7.56	51
	L	135.30	7.23	135	106.84	7.15	107	85.12	7.82	85
ISO 1600	R	134.52	8.50	135	120.79	9.25	121	86.30	10.10	86
	G	140.46	7.79	140	107.10	8.73	108	91.47	10.10	92
	B	109.12	8.42	109	68.30	10.89	69	56.21	9.88	56
	L	135.11	7.91	135	106.81	8.75	107	85.93	9.75	86

R: red; G: green; B: blue; L: luminosity

However, phenolic contents of kombucha fermented with garlic is significantly higher than fermented vinegar (approximately 3 times higher) but the ratio of increase in DPPH inhibition capacity to increased phenolic content in vinegar is higher than in kombucha. While acetic acid is the dominant acid in kombucha [24], Jayabalan et al. [25] also reported gluconic acid, glucuronic acid, vitamins and amino acids content in kombucha; a part of antioxidant activity might be due to the presence of these acids.

3.4. Color analysis

According to color analysis data (Table 2), fermentation of garlic reduces the density of colors and luminosity, but the reductions in garlics fermented in vinegar is more than other samples. So regarding luminosity evaluation, fresh garlic is shinier than fermented bulbs, and kombucha fermented garlic is shinier than vinegar fermented garlic.

Density of red and green color in fresh and vinegar fermented garlics are almost the same and overlapping this two colors express yellowish; however, median of red and green peaks in vinegar fermented garlic are closer to each other; on the other hand, density of blue color in fresh garlic is almost same as red and green, so the yellowish reflect is inconspicuous and overall color appears white, while in vinegar fermented density of blue color has significantly decreased and yellow color is sharper. About kombucha fermented garlic, median of color peaks are far from each other. So the colors are not overlapped. Density of red color is higher than green and blue, also standard deviation for red color is smallest between all other samples, so red color is sharper and overall appearance of bulbs is reddish.

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5. Conflict of interests

We declare that we have no conflicts of interests.

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