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RESEARCH ARTICLE

EVALUATION OF ANTIOXIDANT ACTIVITIES OF KOMBUCHA GREEN TEA.

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Abstract

Background and Aim: Kombucha Green Tea (KGT) a health promoting fermented beverage traditionally made by fermenting a sweetened green tea with a symbiotic culture of yeast species and acetic acid bacteria. This drink is composed of some probiotics such as acetic acid bacteria and lactic acid bacteria and also tea polyphenols, sugars, ethanol, water soluble vitamins, a variety of micronutrients produced during fermentation and organic acids as acetic and gluconic acids which are the main metabolites produced. The broth result from fermentation has beneficial effects such as regulation of gastrointestinal and glandular activities, positive influence on the cholesterol level, toxin excretion and blood cleansing, diabetes, aging problems, arteriosclerosis and it has been thought to be a prophylactic and therapeutic beneficial agent to human health from weight loss to curing cancer. And so, this study aims to use kombucha green tea as a natural remedy as result of its antioxidant and antimicrobial activities.

Methods using: Radical scavenging assays by 2, 2-diphenyl-1-picrylhydrazyl (DPPH), and scavenging hydrogen peroxide assay were used to determine the antioxidant activity of the KGT spectrophotometrically. Evaluation of antimicrobial activity of KGT against different pathogenic bacteria that cause human diseases using disk diffusion method.

Results: Data showed increasing of the antioxidant activity of the fermented KGT gradually within fermentation time reach to the maximum increase observed on the 14th day with slightly decrease at its end. KGT exhibited higher hydrogen peroxide scavenging activity at 14th day of fermentation by 38.8%. increasing of KGT concentration lead to increasing % of inhibition of DPPH within fermentation time that reach to maximum at 14th day obtaining IC₅₀ 0.1196 ± 0.0290 (P < 0.05). KGT obtained clearly antibacterial activity at 14th day of fermentation on *klebsiella pneuonimae* (K55) and on *Escherichi coli* (C95).

Conclusion: this study revealed that the kombucha green tea (KGT) possess great with antioxidant and antimicrobial activities. These findings provide additional support for the traditional use of Kombucha tea in the treatment of metabolic diseases and different types of cancer.

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Introduction:-

Kombucha green tea (KGT) is a slightly sweet and acidic beverage which is prepared by fermenting sweetened green tea with the tea fungus, a symbiotic consortium of acetic acid bacteria and different yeasts known as SCOBY (Kappel and Anken 1993; Sreeramulu et al., 2000). Several lactic acid bacteria (LAB) have been isolated from some Kombucha associations (Greenwalt et al., 2000; Yang et al., 2010; Marsh et al., 2014). KGT fermentation process leads to the formation of a cellulosic pellicle layer floating on the surface of the growth medium. The composition of yeasts and bacteria in the Kombucha symbiotic consortium is highly variable. It showed that The main acetic acid bacteria present in the tea fungus are *Acetobacter xylinum* (Balentine, 1997), *Acetobacter pasteurianus* (Liu et al., 1996) and *Gluconobacter oxydans* (Liu et al. 1996; Greenwalt et al., 2000; Kurtzman et al., 2001).

The yeast cells convert the sugar into ethanol by the glycolysis pathway (Sievers et al., 1995). Acetic acid bacteria convert glucose to gluconic acid and fructose into acetic acid. Acetic acid stimulates the yeast to produce ethanol which facilitates the growth of acetic acid bacteria and then the production of acetic acid (Liu et al., 1996). Both ethanol and acetic acid have been reported to have antimicrobial activity against pathogenic bacteria (Liu et al., 1996).

The benefits that have been attributed to KGT is also mainly due to green tea catechins content which is polyphenol derivative. These substances act as potent antioxidants and protect against the development of diseases. The beneficial effects of tea mentioned below have been studied mainly *in vitro* while others are based on clinical and epidemiological evidence (Valenzuela, 2004). Tea catechins act as antioxidants, due to the fact that they are molecules with a high capacity to scavenge free radicals and even metals which is also known as redox potential.

Kombucha green tea is claimed to help digestion, prevent microbial infection, help in combating stress, give relief from arthritis, cure cancer and AIDS, and increase immunity (Ram et al., 2000). Some of these claims effects have been proven; this drink does exert antimicrobial activity against a range of bacteria (Greenwalt et al., 1998, 2000; Sreeramulu et al., 2000), prevent the angiogenesis of prostate cancer cells (Srihali et al., 2013), prohibits paracetamol induced hepatotoxicity (Pauline et al., 2001), blocks pancreatic alpha-amylase in the small intestine (Kallel et al., 2012) and the activity of glucuronidase an enzyme indirectly related with cancers (Wang et al., 2010).

Kombucha green tea has been ascribed to the presence of phenolic antioxidants (Vijayaraghavan et al. 2000) which depend primarily on the phenolic content of the substrate and secondarily on the normal microbiota of the Kombucha culture which in turn determine the nature of produced metabolites (Jayabalan et al. 2014). These compounds act as antioxidants in the body by scavenging harmful free radicals implicated in degenerative diseases.

The analyses of the fermented liquid proved the presence of acetic, lactic and gluconic acids as the major chemical compounds (Battikh et al., 2013). They have been suggested to be the major antimicrobial agent (Greenwalt et al. 1998) and other compounds such as bacteriocins and tea-derived phenolic compounds may be involved (Sreeramulu et al. 2001). Systematic investigation of the antimicrobial activity of KGT the presence of antimicrobial compounds other than organic acids or proteins (enzymes) produced during fermentation process (Sreeramulu et al. 2001).

Materials and Methods:-**Chemicals:-**

All chemicals and solvents were purchased from Sigma–Aldrich, USA.

Collection of sample:-

Lipton green tea and Sucrose were purchased from local market, Kombucha culture starter (Botany and Microbiology Department, Faculty of Science, Benha University, Egypt).

Preparation of kombucha green tea (KGT):-

Kombucha green tea was prepared according to the method by Malbaša et al. (2011). Sugared water was prepared with 100 grams sucrose per liter of boiled drinkable water. The boiled sugared-water was then infused with 10 grams of Lipton green tea (thread hanging outside) and boiled again for 5 minutes after that the tea bag was

removed. The boiled sugared green tea was transferred into a clean transparent glass jar that had been previously sterilized at 121°C for 20 min and then allowed to cool to room temperature. It was inoculated with 5% (w/v) of freshly grown tea fungus that had been cultured in the same medium for 14 days and 10% (v/v) of previously fermented liquid tea broth aseptically. The fermentation container was then covered with Gauze for aeration that is required for fermentation of the green tea and kept in a clean environment and left to grow at 30°C a period of fermentation.

Hydrogen peroxide scavenging assay:-

The ability of KGT to scavenge H₂O₂ was determined using the method (Ruch et al., 1989). A solution of H₂O₂ (40 mM) was prepared in phosphate buffer (pH 7.4). H₂O₂ concentration was determined spectrophotometrically using D250 plus, Analytic Jena. One mL of KGT was added to H₂O₂ solution (0.6 mL, 40 mM). Absorbance of H₂O₂ at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without H₂O₂. The percentage scavenging of H₂O₂ by KGT was determined as follows:

$$\text{Hydrogen peroxide scavenging activity [\%]} = [(A_0 - A_1) / A_0 \times 100]$$

Where A₀ is the absorbance of the control, and A₁ is the absorbance in the presence of the kombucha green tea at 7th, 14th, 21th and 28th day of fermentation.

DPPH scavenging activity assay:-

The electron donating ability of KGT 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 0.002%. Different concentrations of KGT (0.05, 0.1, 0.2, 0.4, 0.8, 1.2 and 1.6) 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 darkness for 30 minutes. Later optical density was recorded at 517 nm using spectrophotometer D250 plus, Analytic Jena at 7th, 14th, 21th and 28th days of fermentation. Distilled water with DPPH was used as control (Lawrence R and Lawrence K, 2011; Ebrahimi et al., 2016).

$$\text{Inhibition of DPPH activity (\%)} = (A - B / A) \times 100$$

Where, A=optical density of control; B=optical density of sample.

IC₅₀ factor of samples were also evaluated; IC₅₀ factor indicates the concentration or amount of KGT that can inhibit 50% of free radical agent. Samples with smaller needed amount to inhibit 50% of DPPH have higher radical scavenging activity (Ebrahimi et al., 2016). The IC₅₀ is calculated by graphpad prism 7.

Antimicrobial activity of kombucha green tea:-

The antimicrobial activity of the kombucha green tea (KGT) was determined by the disk diffusion method which is based on the spread of antimicrobial compound in solid medium (Lesueur et al., 2007). The Mueller–Hinton agar was poured in sterile petri dishes (90 mm diameter). The sterilized filter paper discs (6 mm diameter) were impregnated with 50µ of KGT against certain bacterial strains included as Escherichia coli (C95), Salmonella typhimurium (ST74), klebsiella pneumonia (K55) and shigelladysenteriae (S151) obtain from the Faculty of Medicine, Benha University at different days of fermentation to determine the best fermentation day that gives highest inhibition zone using unfermented tea as negative control. Petri dishes were allowed to stand for 30 min at room temperature before incubation at 37 °C for 24 h. The effect of kombucha was reflected by the appearance around disc with a transparent circular zone corresponding to the absence of growth. The diameter of inhibition zone was measured in mm. The larger the diameter of the area, the more susceptible the strain was determined (Choi et al., 2006).

Results and Discussion:-

Antioxidant activity:-

There has been a global trend toward the use of phytochemical present in natural resources as antioxidants and functional foods. Kombucha green tea (KGT) is also attributed to its antioxidant activity. Antioxidants are known to decrease or prevent disorders and metabolic diseases made by free radicals (Jayabalan et al. 2014). Hydrogen peroxide assay is one of methods used for determination of antioxidant activities. Kombucha green tea exhibited higher hydrogen peroxide scavenging activity at 14th day of fermentation by 38.8% while 35.59% at 7th day of fermentation slightly decrease 21th and 28th by 37.3% and 36% and this illustrated in (figure 1). The differences in

H₂O₂ scavenging capacities may be attributed to active components which determine their electron donating abilities. The results had a statistically significantly ($p < .05$)

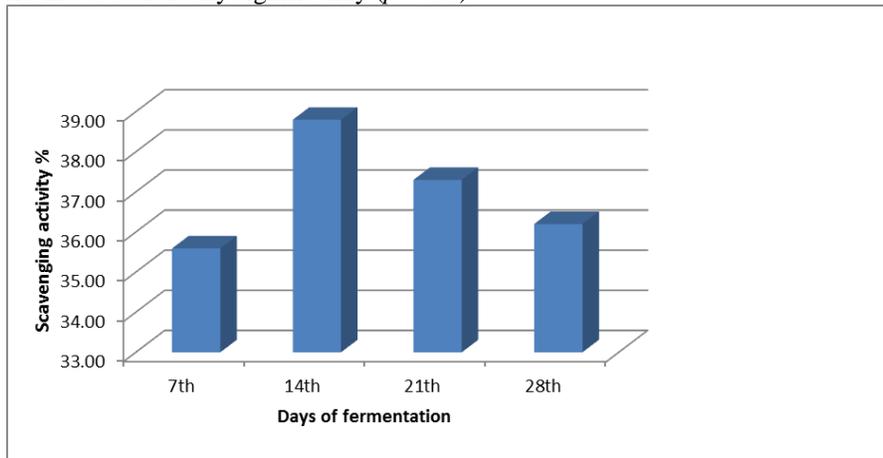


Figure 1:-Comparison of the Hydrogen peroxide scavenging assay of kombucha at different days of fermentation.

The second method for determination of antioxidant activity is DPPH free radical scavenging that was used to determine the concentrations of KGT at which they scavenge the 50% of the DPPH solution termed as IC₅₀ values. The lower the IC₅₀ value of an antioxidant the higher would be its free radical scavenging power. The data showing increasing of KGT concentration lead to increasing % of inhibition within fermentation time that reach to maximum at 14th day of fermentation with slight decrease at 2^{1st} and 28th days of fermentation and this showed at (Figure 2)

The mean IC₅₀ of KGT at the 14th day was 0.1196± 0.0290. The IC₅₀ values of Kombucha green tea are 0.1773 ±0.0227, 0.1523 ±0.0287, 0.1458 ±0.0328 at 7th, 21th and 28th days of fermentation. The comparison fermented KGT at different days showed that the 14th day of fermentation showed the highest scavenging activity for DPPH with mean IC₅₀ value 0.1196±0.02902 and this showed at (Figure3). The results had a statistically significantly ($p < .05$)

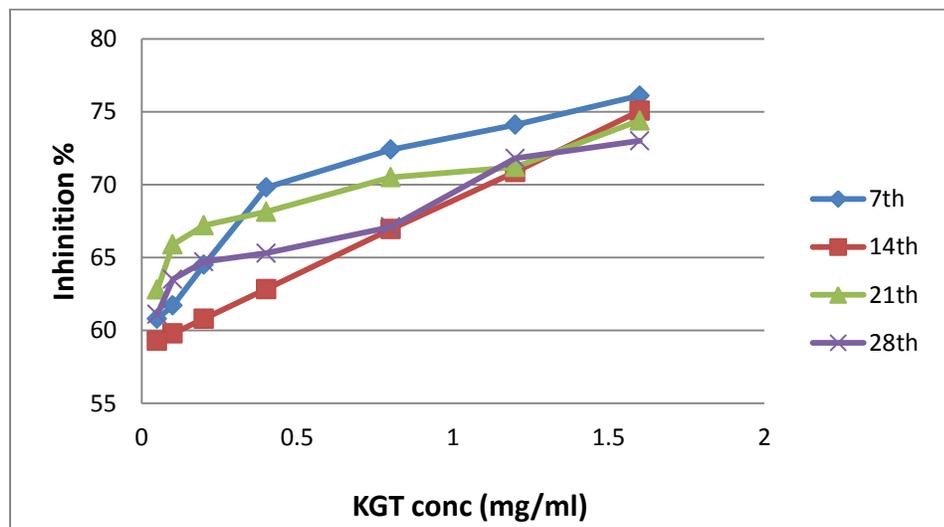


Figure 2:-Comparison of the inhibition % values of kombucha green tea at different days of fermentation.

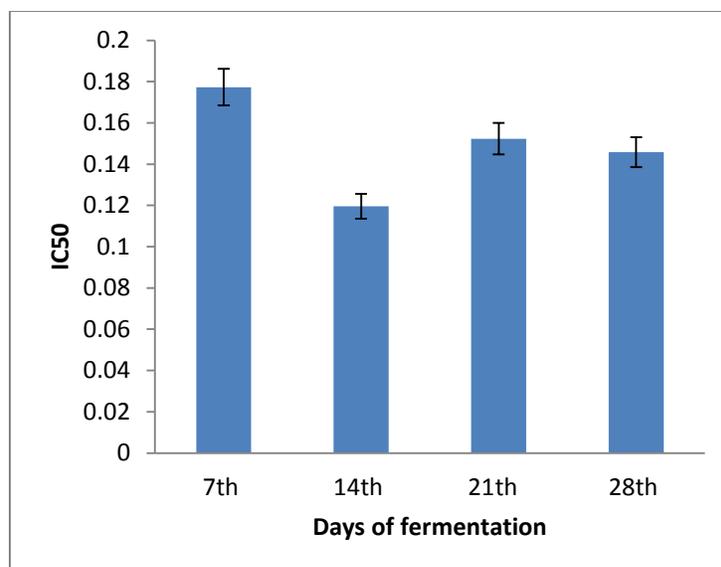


Figure 3:-Comparison of the IC₅₀ values of KGT at different days of fermentation.

The antioxidant activity of kombucha tea is due to the presence of tea polyphenols, ascorbic acid and DSL. Kombucha tea was observed to have higher antioxidant activity than unfermented tea and that may be due to the production of low molecularweight components and structural modifications of tea polyphenols by enzymes produced by bacteria and yeast during fermentation. The extent of the antioxidant activity in the Kombucha green tea beverage depended on many factors such as fermentation period, substrate types and the normal flora of the KGT culture that determined the nature of the secondary metabolites that developed during the fermentation process (Jayabalan et al., 2014). We considered the scavenging effects to be attributable to the sum of the antioxidant ability of many compounds present in the KGT and to the synergistic effects between certain metabolic produced during the fermentation process. Maier et al. (2009) recommended that the antioxidant activity could be a synergic effect among many compounds rather than a single one. Increasing in antioxidant activities may be also due to the peptides result from yeasts during autolysis. Alcaide- Hidalgo et al. (2007) indicate that peptides released by autolysis of *S. cerevisiae* under acidity conditions presented antioxidant activities.

The total content of polyphenols and flavonoids were positively correlated to the antioxidant activity of KGT before and after fermentation. A high polyphenol and flavonoid content are associated with high antioxidant activity (Sun et al. 2015). However, Dani et al. (2010) suggested that the antioxidant activity of phenols and flavonoids have concentration limited and that above this limit the activity cannot increase further with phenolic concentration and so our result obtained in agreement with previous studies. Moreover, Malbařsa et al. (2011) reported that the application of different kombucha starters causes a development of different antioxidant activities on both substrates.

Antimicrobial activity:-

In the present study, the antimicrobial activity of kombucha green tea was screened against various pathogens in vitro by disk diffusion method. The result shown at table (1) and figure (4) is the antibacterial activity of KGT against gram negative enterobacteriaceae. The table indicates that the best antimicrobial activity occurred at 14th day of fermentation. KGT has effect on *klebsiellapneumonimae* (K55) by 21 mm at 14th day of fermentation with no change at 2nd period of fermentation. In addition, appear an effect on *Escherichi coli* (C95) (10 mm) at 14th day of fermentation. And also there is no effect of KGT on *shigelladysenteriae* (S151) and *Salmonella typhimurium* (ST74). Unfermented kombucha green tea on tested bacteria showing no activity.

Table 1:-Antimicrobial activity of KGT within fermentation.

Inhibition zone (mm)					
Tested organism	7 th day	14 th day	21 th day	28 th day	Unfermented tea
klebsiellapneumonimae (K55)	14	21	21	20	0

shigelladysenteriae (S151)	0	0	0	0	0
Salmonella typhimurium (ST74)	0	0	0	0	0
Escherichi coli(C95)	0	10	10	9	0

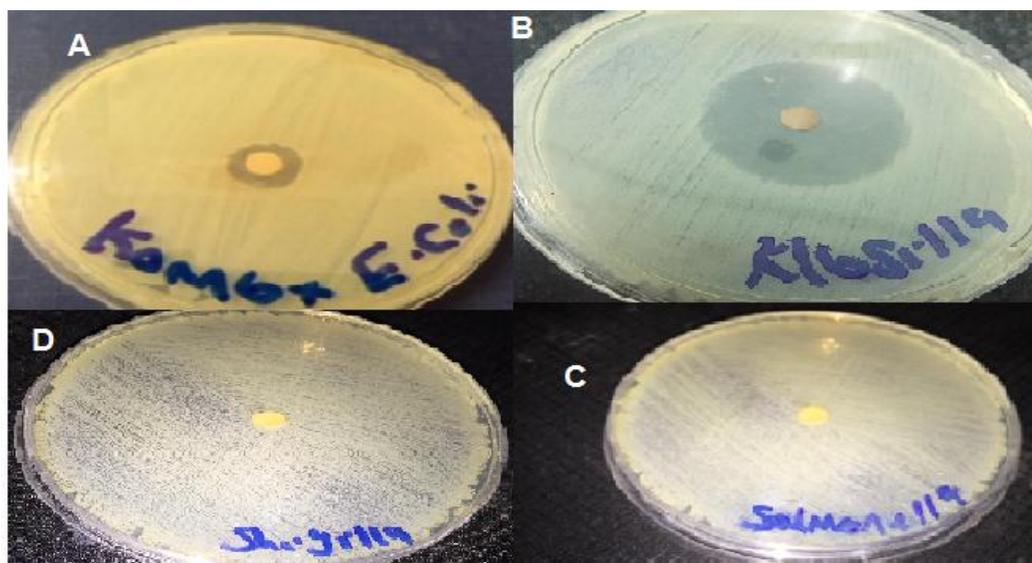


Figure 4:-Antimicrobial activity of unfermented kombucha green tea, A: Escherichi coli, B: klebsiellapneumonimae, c: Salmonella typhimurium and D: shigelladysenteriaeat 14th day of fermentation.

Enteric bacterial infections are a major disease burden in human population in developing countries. Earlier these infections could be treated with low-priced antibiotics. However, recently the treatment has become higher cost with less successful due to the presence of multi-drug resistance among the enteric strains such as E. coli, Salmonella spp and Shigella spp (Barman et al., 2010). Hence, it has become imperative that the problem of multidrug resistance is battled at the earnest by exploring medicines or products from natural sources as a potential source of novel antimicrobial agents against the resistant bacteria (Ross et al., 2001).

The inhibition to E. coli and klebsiella pneumonimaeare important because this enteropathogenic microorganism is responsible for diarrheal disease and nosocomial infections that still the most prevalent and important public health problem in developing countries which is mainly cause death in children with less than 5 years of age, despite advances in knowledge, understanding and management that have occurred over recent years (Fagundes Neto and Scaletsky, 2000).

The Kombucha green tea showed also remarkable antimicrobial activity against E. coli (C95) and klebsiella pneumonimae (K55) where antimicrobial activity increased with fermentation time with slightly changes at 2nd period of fermentation. There are numerous reports that antimicrobial activity of kombucha tea against pathogenic microorganisms is largely attributable to acetic acid (Greenwalt et al., 1998 ; Battikh et al., 2013) and antibiotics present in the kombucha tea (Chen and Liu, 2000; Jayabalan et al., 2007). More, Sreeramulu et al. (2001) reported that the metabolites produced by the bacteria and/or yeasts during the fermentation of kombucha tea are responsible for its antimicrobial activity. Our results are similar to previous studies.

Conclusion:-

This study revealed that the kombucha green tea (KGT) possess great with antioxidant and antimicrobial activities.

Recommendation:-

These findings provide additional support for the traditional use of Kombucha green tea (KGT) in the treatment of metabolic diseases and different types of cancer.

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