Formulation, Preparation, Physico-chimical Analysis, Microbiological Peculiarities and Therapeutic Challenges of Extractive Solution of Kombucha

OLIMPIA DUMITRIU BUZIA, VALERIA FASIE, NELA MARDARE*, CAMELIA DIACONU, GABRIELA GURAU, ALIN LAURENTIU TATU

Dunarea de JosUniversity of Galati, Faculty of Medicine and Pharmacy, Research Center in the Field of Medical and Pharmaceutical Sciences, Pharmacology Sciences Department, 35 Al. I. Cuza Str., Galati, Romania

This paper aimed at the preparation and characterisation of an extractive solution of Kombucha. We have applied the method of water extraction of a species of yeast, Kombucha, grown and proliferated in the pharmaceutical technology laboratory of the Faculty of Pharmacy at Dunarea de Jos University of Galati. Samples of various sugar concentrations have been prepared, as well as samples subject to concentration, thus obtaining extracts of up to 80% concentration. The extract obtained was analysed chemically and microbiologically. The main elements highlighted in the composition of the extractive solution were: iron, zinc, magnesium, calcium and potassium. The antimicrobial activity in strains: Gram-positive: Staphylococcus aureus, Gram-negative: Pseudomonas aeruginosa, Escherichia Coli, fungi: Candida albicans.

Keywords: kombucha, extract, pseudomonas aeruginosa, candida albicans, staphylococcus aureus

During the Tsin dynasty,[1](2000 years ago), a yeast was discovered, being considered the mysterious *nepenthe* of *eternal life*. It is a form of life which grows in an environment obtained by infusing sweetened black tea; it grows fast as a gelatin mass, and the watery, yellowy extract obtained has a fresh and very pleasant taste. A few hundreds of years later, it was made official as medicine in Japan, during the rule of Emperor Inkyo. The yeast was brought by merchants from Far East to Russia. It started to be studied in 1915, especially in Russia, Germany and the Baltic countries. During the same period, the extract became a very popular traditional medicine [2]. It is used from the Ural Mountains to Switzerland or Spain. It is a colony of 12 to 20 species of microorganisms (much more, according to some researchers) which live in symbiosis, making up a complex biological system, fascinating through its organisation.

The colony consists of *friendly* bacteria and yeasts which transform the sugar they grow in, as well as the active principles of black or green tea in substances with remarkable curative effects. Kombucha is an effervescent beverage with sweet-sour taste, resembling cider's [3]. Kombucha is a living elements, and just like fruit or vegetables, contains enzymes beneficial for the digestive process. It also stimulates the organism's natural resistance.

Experimental part

Materials and methods

Kombucha yeast (purchased from a private person from Chisinau), which we proliferated and analysed.

Green, black, white or oolong tea, with which the watery extract was made; sugar, starter liquid (yeast or SCOBY) (table 1). Reference cultures and cultures collected from patients of *Staphylococcus aureus*; turbidity of inoculum measurement apparatus, test tubes, microcomprimates, culture environment with M.H agar (Muller Hinton) for Gram-negative bacilli and S. aureus and Sabourad environment for fungi, using the Kirby-Bauer diffusion test.

Table 1
CONCENTRATION IN SUGAR OF THE SAMPLE ANALYSED AND FERMENTED

Nr.crt.	G sugar/l (green tea)	Days-fermentation	Extract fluid
1	100	10	80%
2	75	7	80%
3	50	7	80%
4	80	10	80%
5	75	11	80%
6	100	10	-
7	75	7	-
8	50	7	-
9	80	10	-
10	75	11	-

For the spectrophotometric dosage of the ions detected in the watery extract, we used a number of reactive chemicals, such as Xylenol orange, acetate buffer, sodium acetate 25%, Hydroxyl ammonium chloride 10%, O-Phenanthroline in ethyl alcohol 0.5%.

The Kombucha extract is obtained this way: first, an infusion [4.5] of green tea is made (4 g green tea in 1 liter of water) and 100 g of sugar. It is filtered through a sieve, cooled, and then placed in a wide mouth jar. A gelatin layer of Kombucha is then added in the jar, together with some yeast (depositions from the bottom of the bowl) from an old culture; the jar is kept for 7-11 days at optimal temperature between 23-27°, in which time fermentation takes place, then it is filtered. This time is necessary for the friendly microorganisms in this colony to consume the sugar and transform it in curative substances. The remaining gelatine mass is used for new cultures. Although the chemical analysis of the yeast has not been completely elucidated, it is already known that it contains large quantities of vitamin C, as well as B-complex vitamins (B1, B2, B3, B6, B12), which justifies, to some extent, its rejuvenating properties and its stimulating effects for the

* email: hipocratgalati@yahoo.com *All authors had equal contribution to designing and writing the presented paper.*

organism's natural resistance. In this solution, we have also discovered very strong antibiotic and antiviral substances, which explains its successful use in the treatment of infectious diseases [6].

The chemical composition of Kombucha beverage is the following:

4-Acetamidophenol, 4-Acetamidofenol, acetic acid, aceto-acetic acid, benzoic acid: 2-amin0-, 3-phenyl-2-esther prophenyl, butyric acid, 3-methyl, benzonitrile, 4-hydroxy-2(4H)-Benzofuranone, 5,6,7a-tetrahydro-4,4,7a-trimetyl-2,6-Bist(t-butil)-4-(dimetilbenzil) phenol, 1-Butanol, 3-methyl-2-t-Butil 4-(dimethyl benzene) phenol, carbonic acid, citric acid, caffeine, cobalamine, cyanocobalamin, decanoic acid 2,3-Dihydro-1-metilindena 2,5 Diketo- gluconic acid, D-Acid gluconic D-Ribo-hexos, 2,6-dideoxy-3-0-metyl-, D-acid saccharin (glucaric acid), D-acid saccharin 1,4 lacrone (Glucaro 1,4 lactone), D- acid xylonic, glucuronic acid Ethyl acetate, enzymes; a great variety, folic acid, glucose, hexanoic acid (1H)mdazo[2,1-f]purine-2,4(3H,8H)-dione,8-ethyl-1-methyl-7-phenyl-, itatonic acid 2-Keto-acid gluconic,5-Keto-acid gluconic,2-Keto-deoxy-acid gluconic, lactic acid, 5-Metoxi-1-(3-metoxy-4-methylphenyl)-1,3,3,6-tetramethylindan niacin, Niacinamid, nicotinic acid, octanic acid, Acid oxalic acid, pantothenic acid, alcohol fenetil, phenol,4-etil,6-Phospho gluconate, malonic acid, propionic acid, Pyridoxine, Riboflavin, Acid succinic acid, tartaric acid, Thiamine, usnic acid. There have also been identified other 40 traces of various acid types: amino-acids: (lysine, alanine, tyrosine, valine, phenylalanine, leucine isoleucine, aspartic acid, glutamic acid, serine, threonine etc.)[7].

We have made chemical determinations in view of detecting the main elements in the composition of the extract. Spectrophotometric determinations have been made on the six samples in different moments of fermentation, in view of highlighting the presence of the main ions: iron, zinc, calcium, magnesium, etc. The results obtained are presented in table 2.

Table2

VARIATION OF Fe AND Zn CONCENTRATION BASED ON SUGAR CONCENTRATION AND THE NUMBER OF DAYS OF FERMENTATION

№ crt.	Sugar concentration/l extractive solution	Days of fermentation	Fe mg/l	Zn mg/l
1	75g/l	7	0.4268	0.2193
2	75g/l	11	0.5608	0.2613
3	100g/l	10	0.5432	0.2733
4	80g/l(water)	10	0.1718	0.0718
5	50g/l	7	**1.1086**	0.1474
6	50g/l	11	0.8315	**0.4598**

Results and discussions

Quantitative determination of the iron ion

To obtain the standard stairs I used stock solution $NH_4Fe(SO_4)_2*12H_2O$,what contains at 1,000 mL stock solution a quantity of 0.1 mg Fe^{2+} ; were prepared 5 samples at which they were measured absorbances at a wavelength λ= 510nm.

The concentrations of the 5 samples for the standard scale and their abilities are shown in the table 3.

Table 3

CONCENTRATION AND ABSORBANCE OF Fe

Sample	1	2	3	4	5
Concentrations obtained (mg Fe^{3+})	0.8	1.6	2.4	3.2	4
Absorbance (nm)	0.132	0.274	0.430	0.570	0.718

Depending on the concentration of the standard solutions and the absorbances obtained at each of them, we will obtain the calibration curve for the Fe^{2+} and the equation of the straight line (fig.1)in which we will interpol the absorbances obtained for each sample from the Kombucha solution.

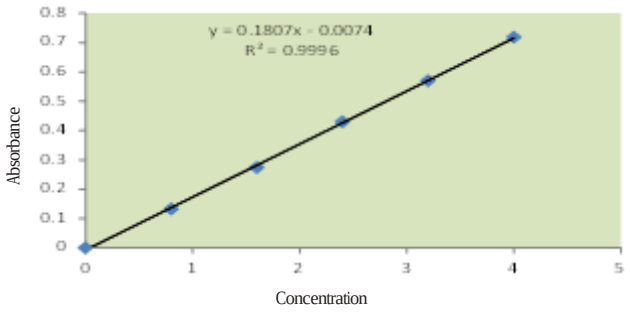


Fig. 1. Standard scale for Fe^{2+}

For calculating the concentration Fe^2z in the samples to be analyzed we use the equation of the straight line,obtained on the standard scale for Fe^2:

$$y = 0.1807x - 0.0074$$

$$x = \frac{y + 0.0074}{0.1807}$$

$$y = Absorbance$$

$$x = Fe^{2+}concentration$$

Absorbents read at λ=510nm and concentrations in Fe^{2+} obtained for the analyzed samples are as follows (table 4).

Depending on the Fe^{2+} concentrations in the analyzed samples we can interpret that iron is found in higher amounts in the less sugar samples(samples V and VI) in the medium used for feeding the fungus; and in the sample

Sample	I	II	III	IV	V	VI
Absorbance (la 510 nm)	0.070	0.094	0.091	0.024	0.193	0.143
Concentration (mg/L)	0.4268	0.5608	0.5432	0.1718	1.1086	0.8315

Table 4

ABSORBENTS READ AT λ=510 nm AND CONCENTRATIONS IN Fe^{2+}

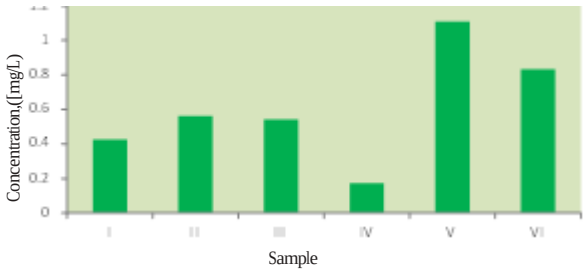


Fig. 2. The variation of Fe^{2+} the analyzed samples

containing water instead of the infusion of green tea the amount of iron is lower (table 4).

Quantitative determination of the zinc ion

To obtain the standard scale is used stock solution $ZnSO_4 * 7H_2O$ containing at 1000mL stock solution, an amount of 1mg Zn^{2+}; 5 sample were prepared at which the absorbance were measured at a wavelength λ=580nm

The concentrations of 5 samples for standard scale and their absorbents are shown in the following table 5.

Table 5

CONCENTRATION AND THE ABOSORBANCE OF STANDARD SCALE FOR Zn^{2+}

Sample	1	2	3	4	5
Concentrations (mg Zn^{2+})	0.2	0.6	0.8	1.2	1.6
Absorbance (nm)	0.079	0.156	0.286	0.423	0.531

Depending on the concentration of etalon scale and the absorbances obtained at each of them, we will obtain thestandard curve of Zn²z and the equation of the straight line (fig. 3) in which we interpolate the absorbances obtained for each Kombucha solution

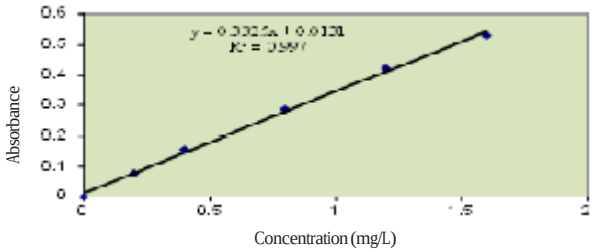


Fig.3. The standard curve of Zn^{2+}

To calculate the concentration Zn^{2+} in the analyzed samples we use the equation of the straight obtained on the standard scale of zinc:

$$y = 0.3325x + 0.0131$$

$$x = \frac{y - 0.0131}{0.3325}$$

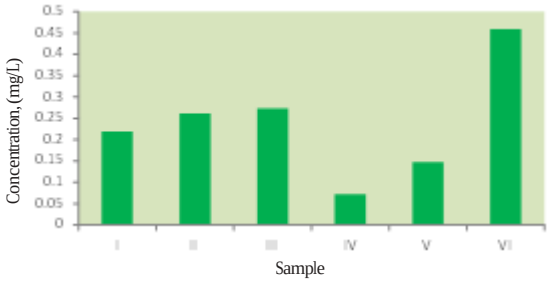


Fig. 4. Variation of zinc ions

The absorbances read at λ=580nm and the Zn concentrations obtained for the analyzed samples are presented in table 6.

Depending on the Zn^{2+} ion concentration in the analyzed samples, we can interpret that zinc is found in a larger quantity in the sample with the smallest amount of sugar and with a large number of fermentation days(sample VI); and in the sample containing water instead of infusion of green tea, the amount of zinc is lower (table 2).

We can conclude that, in the case of iron, the concentration increases in low quantities of sugar (50g/L) and 7 days of fermentation. The daily iron requirement is of 15 mg/day for male and female adults [8]. The iron concentration in the solution analysed is, therefore, significant and can be an important natural source.

In the case of zinc, the concentration increases in low quantities of sugar (50 g/L) and has high variations in the days of fermentation (11 days). It is a mineral with multiple therapeutic valences, proven effective in: immunity increase, cold, macular degeneration, healing of cutaneous lesions, infertility, high cholesterol, ADHD, blepharitis, dermatitis, psoriasis, herpes simplex, various alopecia, etc. [9].

Ca^{2+} and Mg^{2+} dosage

For the dosage of calcium and magnesium, we used the complexometric method on the extract obtained from green tea and sugar, as shown in table 7.

The *p*H of the solutions obtained varied from 2.5 to 4, based on sugar concentration and especially on the days of fermentation.

Sample	I	II	III	IV	V	VI
Absorbance (nm)	0.086	0.100	1.104	0.037	0.180	0.166
Concentration (mg/L)	0.2193	0.2613	0.27338	0.0718	0.1474	0.4598

Table 6

THE ABSORBANCE READ AT λ=580 nm AND THE Zn CONCENTRATIONS

Nr.crt.	Sugar concentration/l extract	Days of fermentation	Calcium	Magnesium
1	75g sugar/l	7	4.8mg/dl	**3.7mg/dl**
2	75g sugar /l	11	**5.6mg/dl**	1.75mg/dl
3	100g sugar /l	10	4.008mg/dl	0.972mg/dl
4	80g sugar /l water	10	4.4mg/dl	1.7mg/dl
5	50g sugar /l	7	4.2mg/dl	2.3mg/dl
6	50g sugar /l	11	4.6mg/dl	0.83mg/dl

Table 7

VARIATION OF THE Ca AND Mg CONCENTRATION BASED ON SUGAR CONCENTRATION AND THE NUMBER OF DAYS OF FERMENTATION

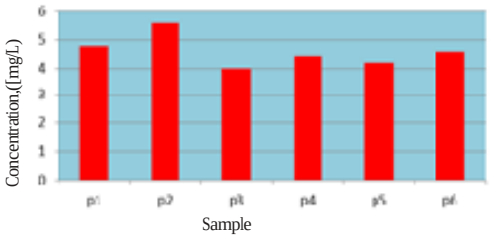


Fig. 5. The variation of Ca^{2+} in the analyzed samples

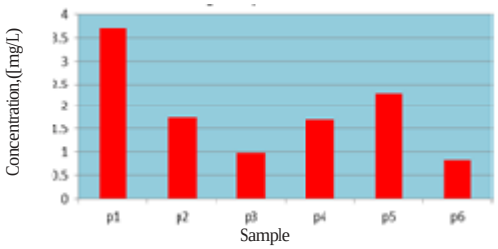


Fig. 6. The variation of Mg^{2+} in the analyzed samples

Nr.crt	g/sugar/l green tea extractive solution	Days of fermentation	Fluid extract
1	100	10	80%
2	75	7	80%
3	50	7	80%
4	80	10	80%
5	75	11	80%
6	100	10	-
7	75	7	-
8	50	7	-
9	80	10	-
10	75	11	-

Table 8
SAMPLES FOR ANALYSIS (FROM 1-5- FLUID EXTRACT; FROM 6-10 WATERY EXTRACT SOLUTION)

Nr.crt	G sugar /l green tea	Days of fermentation	fluid Extract	SA Reference	SA Patients	EC Ref.	EC Pat.	PA Ref.	PA Pat.	CA Ref.	CA Pat.
1	100	10	80%	12	13	10	**11**	9	12	15	11
2	75	7	80%	12	13	11	10	6	9	12	**12**
3	50	7	80%	13	**16**	8	8	9	6	10	9
4	80	10	80%	7	-	7	-	8	7	-	-
5	75	11	80%	11	11	14	7	12	**13**	9	9
6	100	10	-	11	-	10	-	-	-	-	-
7	75	7	-	13	-	10	-	-	-	-	-
8	50	7	-	12	-	8	-	-	-	-	-
9	80	10	-	-	-	-	-	-	-	-	-
10	75	11	-	12	-	10	-	-	-	-	-

Table 9
INHIBITION DIAMETERS OBTAINED PER REFERENCE CULTURE AND CULTURES FROM PATIENTS WITH SA,EC,PA,CA

We can conclude that, in both cases, the concentration increases with the quantity of sugar and the days of fermentation.

Calcium and magnesium are essential minerals for the organism; we need calcium and magnesium for healthy teeth and strong bones, for the rhythmic functioning of the heart and blood circulation, for the effective functioning of the nerves and muscles. More than 99% is found in bones, but it also has an important role in muscular contraction, heart functioning and coagulation [10].

Conclusions

- at a small quantity of sugar, we will have more Fe and Zn;
- at a higher quantity of sugar, we will have more Ca and Mg;
- for a small fermentation period (7 days), we will have more Fe
- for a longer fermentation period (11 days), we will have a higher quantity of Zn, Ca and Mg.

The paper underlines the antibacterial action of the watery Kombucha solution in black tea and of the fluid extract of Kombucha over standard strains and strains from patients with Staphylococcus aureus (SA), Pseudomonas Aeruginosa (PA), Escherichia coli (EC) and Candida albicans (CA).

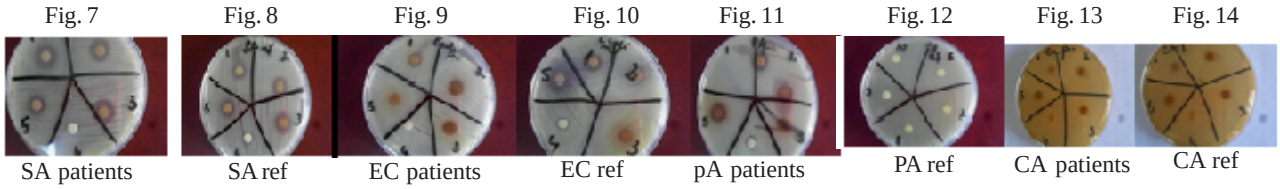
- reference and patient cultures of staphylococcus aureus
- apparatus for measuring the turbidity of inoculum;
-test tubes, microcomprimates, culture environment with cu M.H. agar

The working technique used was the diffusimetric method: on a plate of M.H. agar, the inoculum was placed (in 3 mL physiologic serum the bacterial culture to be analysed is inoculated up to a 0.5 McF turbidity, so that it covers the entire surface of the environment, in three directions:

It is left 10-15 for the absorption of inoculum on the environment surface, then the sterile microcomprimates are added, soaked in the solution to be analysed (fluid Kombucha extract -samples 1-5 and watery solution - samples 6-10, respectively) at 3 cm distance from one another and at 1.5 cm from the edge.

It is then left 10-15 MIN for the microcomprimates to adhere to the agar surface, they are turned and left at the thermostat at T=37°C for 18-24 h.

The inhibition diameter for each sample is then measured.



For samples 4 and 9, we used water boiled at 100°C instead of green tea. The 80% represents the fluid extract obtained from Kombucha green tea.

Figures 7-14 show the inhibition areas of our extractive solution over: Staphylococcus aureus (SA) from cultures from patients (fig. 7) and reference culture (fig. 8), Escherichia Coli (EC) from cultures from patients (fig. 1. Standard scale for Fe^{2+}9) and reference culture (fig. 10), Pseudomonas Aeruginosa (PA) from cultures from patients (fig. 11) and reference culture (fig. 12), Candida albicans (CA) from cultures from patients (fig 13) and reference culture (fig. 14).

We can conclude that the best results were obtained using the Kombucha fluid extract, which had significant inhibitive areas on Staphylococcus aureus (16) at low sugar concentration (50g/L) and 7 days of fermentation, Pseudomonas aeruginosa(13) at a relatively low sugar concentration (75g/L) but at a maximum 11 days of fermentation, Escherichia Coli (11) at high sugar concentration (100 g/L Kombucha green tea), with 7 days of fermentation and Candidei Albicans (13) at low sugar concentration (75g/L), and 11 days of fermentation.

Therefore, we can successfully use the Kombucha fluid extract in green tea as follows: at low sugar concentration, the best results are obtained for: Candida albicans, Staphylococcus aureus and Pseudomonas aeruginosa, and at high sugar concentration we have the best results for Escherichia Coli.

In what concerns the days of fermentation, there have been recorded maximum results for Staphylococcus aureus and Escherichia Coli after 7 days, and for Pseudomonas aeruginosa and Candida albicans, after 11 days of fermentation.

The paper has partially highlighted the chemical composition and microbiological activity of the Kombucha fluid extract and extractive solution in green tea. The quality of its minerals proves the beneficial effect of this extractive solution for the human organism. In addition, the microbiological determinations made have underlined the role as a natural antibiotic of the extractive solution, which opens the path to novel research in the future.

The topical application of the Kombucha fluid extract might be used in the following clinical studies on the control of carriage areas of staphylococcus aureus: the nasal cavity, the auricles, the axillary and inguinal areas, as well as on the prophylaxis or its adjuvant role in treating the cutaneous-mucous candida infections. Also, the spectrum of its usage may expand towards the acceleration of the cutaneous healing processes in chronic leg ulcers, emerging from chronic venous insufficiency (through decompressed hydrostatic varicose or postthrombotic syndrome), in neurotrophic ulcers, especially when complicated by the presence of strong germs, such as Staphylococcus Aureus, E. Coli or Pseudomonas Aeruginosa. Its use in combination with the specific etiologic treatment, with systemic or topical antibiotics therapy may improve the granulation, epidermisation, antisepsis processes, through the trace elements it contains and also through its antibacterial inhibitor role.

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