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RESEARCH ARTICLE

CHARACTERIZATION OF MICROBIOLOGICAL AND PHYSICOCHEMICAL PROPERTIES OF READY TO DRINK JUICE ENRICHED WITH ARTEMISIA ANNUA

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Abstract

Artemisia annua is used to prepare a tea for the treatment of malaria, but this traditional method was not diffused around the world. This might be due to time to prepare the tea and its taste. Therefore, the aim of this study was to develop and characterize a ready to drink (RTD) pineapple juice enriched with Artemisia annua. RTD juice was obtained by the infusion of Artemisia annua leaves and stems in pineapple juice (100%) or in 75% pineapple juice and 25% water. Microbiological analysis was explored following the method of AFNOR. The chemical composition, the phytochemical and physicochemical properties of RTD juice were performed using standard colorimetric methods. The identification of Artemisinin was done using high performance liquid chromatography. The two RTD juices produced showed a satisfactory microbiological quality. Chemical analysis revealed the presence of vitamin C, which varies from 14.30 mg/100g ± 0.12 to 4.10 mg/100g ± 0.10, coumarins, reducing sugars also a pH which varies from 4.07± 0.09 to 4.20 ± 0.10 in both RTD juices. The Brix degree of both RTD juices varied from 10.70 ± 0.08 to 16.60 ± 0.10°Brix. The (HPLC) revealed a peak of artemisinin in both RTDjuices. The data obtained from this study showed that these two RTD juices contain bioactive substances and might be used to treat malaria.

Introduction:-

Malaria is the most prevalent parasitic disease in the world. In 2018, about 230 million of persons were affected and around 400000 of them were dead. Africa remains the most affected continent, with around 213 million sufferers (≈ 92.6%) and 380,000 deaths (WHO, 2019). The treatments recommended by the WHO are Artemisinin-based Combination Therapies (ACTs) (N'Guessanet al., 2019). However, in addition to their relatively high cost, these drugs are subject to numerous cases of therapeutic resistance, forcing researchers to consider other alternatives (Elfawalet al., 2015; Nsanzabana, 2019). Indeed, several studies have demonstrated the therapeutic efficacy of

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Artemisia annua leaves and stems on CTA-resistant forms of Plasmodium (Daddy et al., 2017). That plant, which grows easily in tropical countries, could provide a solution to the problem of accessibility to cheaper, good-quality antimalarial treatments for people in poor countries (Aftab et al., 2013). However, no drug based on the powdered leaves and stems of Artemisia annua has yet been placed on the pharmaceutical market. A few formulation trials of solid or liquid oral forms (capsules, herbal tea and tablets) have produced good results (Onimuset al., 2013; Sanogo,2021). According to Lutgen (2019), the bioavailability of artemisinin contained in herbal tea is twice than that of CTA pills. That means at the same dose, artemisinin is found to be at least twice more in blood plasma. Despite thebenefit related to the Artemesiaannua, its use around the world should have to increase, but it still be considered as a prophylaxis for poor people (Ogwang et al 2011). The use of Artemesiaannua is still limited to the preparation of an infusion (Rath et al., 2004). This needs around 30 minutes and it might be a problem for some people, in particular, in developed country where people use to reduce the time spent for cooking (Verbeke&Poquiviqui Lopez2005; Buckley et al., 2007,). Other inconvenience that can reduce the use of Artemesiaannua as a prophylaxis might be associated to the bitter taste of the Artemesiaannuatea. For that reason, people use to add different sweetener ingredients as sugar, honey (Ogwang et al., 2012). Therefore, the aim of this study was to develop and explore the microbiological and physicochemical properties of a ready to drink (RTD) pineapple juiceenriched with Artemisia annua.

Materials and Methods:-

Materials:-

The leaves and stems were procured in October 2021 from an Artemisia annua cultivation garden in Segbé (Prefecture of Golf,Togo), and identified in the Botany and Plant Ecology laboratory of the Faculty of Sciences of the University of Lomé. Plant organs were cut into small pieces, washed then dried in the laboratory under air conditioning at 20°C for 15 days (Sanogo, 2021). Pineapple used in this study was the Brazza. These pineapples were obtained from a local supplier in Gbatopé (Prefecture of Zio, Togo).

Methods:-

Production of raw pineapple juice:

Pineapple juice was produced according to the method of Chadaré et al. (2021). After the reception, the pineapple was weighed and sorted to eliminate unripe and rotten fruit. After that, the crowns of the fruits were removed, and then followed by their washing. Washed pineapple was peeled and cut into small pieces then pressed using an electric press to obtain raw pineapple juice. This raw juice was filtered to remove the pulp.

Preparation of the ready to drink juice

The preparation of RTD juice was carried out according to the modified method of Räth et al. (2004). Normally, 5g of dried leaves and stems were infused in one litter of boiling water. In this study, 1/3 of the litre of boiling water or boiling pineapple juice was used to infuse 5g of dried leaves and stems per 10 minutes. Then, the pineapple juice was used to complete the 2/3 of the litre, in manner to reach one litre. This method was used in manner to avoid the alteration of the nutritional component of the pineapple juice. The product obtained with boiling water was named water pineapple juice (WPJ) and the second with boiling pineapple juice was named pineapple juice (PJ). The obtained RTD juice was filled into 33 cl bottles, which were pasteurized at 70°C, 80°C, 90°C for 10, 15 and 20 min. Both RTD juiceswere used for microbiological and physicochemical analyses.

Microbiological analysesof RTD juices

The microbiological evaluation of the RTDjuice was exploredwith the routine standardized methods of the French Association for Standardization based on the KEBS criteria relating to fruits juices (Table 1) (NF V08-051, 1979). Total mesophilic aerobic flora, total coliforms, anaerobic sulfate-reducing germs, yeasts and moldwere counted by the mass seeding technique. Staphylococcus aureus was enumerated by plating following the routine method NF V08-057-1 (1994). Number of colonies was expressed in Colony Forming Unit per ml (CFU/ml) according to the AFNOR standardized formula below.

$$\mathbf{N} = \frac{\Sigma \mathbf{C}}{\mathbf{V(n1+0.1n2)d}}$$

with: **N**: the number of Colony Forming Units per ml (CFU/ml), $\boldsymbol{\Sigma C}$: the sum of colonies counted on all the plates retained, **v**: Volume of the inoculum, **n1**: the number of plates retained at the first dilution, **n2**: the number of plates retained at the next dilution, d: the dilution rate of the first dilution.

Physicochemical analyses of RTD juices

P^H was measured according to the AOAC method (1995) using an OAKTON pH 700 pH meter calibrated with pH 7 and 4 buffers. The pH was obtained by introducing the pH meter electrode into a sample. Then it was rinsed with distilled water before another measurement.

The soluble dry matter, expressed as Brix degree (°Brix), of the RTDjuice was determined by the AOAC method (1995) using a PAL-1 digital refractometer (ATAGO brand). A few drops of juice were placed on the glass part of the refractometer then after pressing the "Start" button on the device, the °Brix value appear directly on the device screen.

Vitamin C content was measured using the modified method of Deymie et al (1981). 10 ml of the sample were pipetted into an Erlenmeyer flask, 10 ml of the extraction solution (the volume/volume mixture of 3% metaphosphoric acid and 8% acetic acid) was added to the sample. The mixture was homogenized and then stored for 30 min. Finally, using a burette, the mixture is dosed with a 0.01N iodine solution (I_2) in the presence of 0.5ml of 0.5% starch.

The quantity of vitamin C (Vit C), expressed as mg of ascorbic acid in 100 ml of juice, was determined by the following formula:

$$\text{Vit C (mg / 100 ml)} = Veq * 8.806 * 10 / V$$

with: Veq: Volume in ml of iodine 0,01N at the equilibria, V: Volume of samplein ml,

The identification of the main chemical groups present in the extracts was carried out and assessed qualitatively from standard colorimetric tests using Odeja et al., (2014)method.

Identification of tannins:

2 ml of water were added to 2 ml of extract contained in a test then one or two drops of 1% ferric chloride (1g of $FeCl_3$ + 65 ml distilled H_2O). A blue, blue-black or black coloration indicates the presence of gallic tannins, whether a green or dark green coloration indicates the presence of catechin tannins.

Identification of saponins:

The extract was dissolved in distilled water and distributed into two tubes, where one was considered as control. Few drops of distilled water were added in the second tube containing2 ml of extract. The appearance of persistent foam of 1 to 2 cm after stirring indicates the presence of saponins.

Identification of triterpenes:

The extract was dissolved in distilled water and distributed into two tubes, where one was considered as control. Few drops of chloroform and sulfuric acid (H_2SO_4) were added in the second tube containing 2 ml of extract. The appearance of a red-brown ring between two phases, one clear at the bottom and the other a little or slightly green at the top indicates the presence of triterpenes and sterols in the extract.

Identification of flavonoids:

The extract was dissolved in distilled water and distributed into two tubes, where one was considered as control. Few drops of methanol and magnesium turnings were added in the second tube containing 2 ml of extract. Then a few drops of concentrated hydrochloric acid (HCl) were added. The appearance of a red colour indicates the presence of flavonoids in the extract.

Identification of coumarins:

The extract was dissolved in distilled water and distributed into two tubes, where one was considered as control. In the second tube, 2ml of distilled water and a small quantity of ammonia (instead of soda) were added to the mixture. The appearance of fluorescence after ultraviolet illumination indicates the presence of coumarins in the extract.

Identification of free reducing compounds:

Fehling liquor reduction test: 2ml of the extract was introduced into a tube, then 2 ml of Fehling's liquor were added. The mixture was boiled in a water bath for at least 30 minutes. Obtaining a brick red precipitate indicates the presence of reducing compounds in the extract

Identification of alkaloids:
One drop of Dragendorff was added to2 ml of extract. The formation of a red-orange precipitate indicates the presence of alkaloids.

Identification of artemisinin:
Artemisinin extraction was performed using a modified method of Räth et al. (2004). 200 ml of each RTD juice was extracted twice with 200 ml of petroleum ether at 60–80°C. The organic phase was dried with sodium sulphateandand the residue was dissolved in 10 ml of ethanol. HPLC analysis of Artemisia leaves was performed using an Agilent Technologies 1100 equipped with a diode array detector. An Agilent Eclipse XDB-C18 column (150 mm L × 4.6 mm, particle size of 4 µm) was used with a mobile phase composed of phosphate buffer pH = 6.3 (solvent A) and methanol (solvent B) in the proportions of 55 and 45%, respectively, pumped using a pump. Analysis was performed in isocratic mode with a flow rate of 1 ml/min and the analysis time was 15 min. Detection was done at a wavelength of 260 nm.

Data elaboration:
The collected data was elaborated in a Microsoft Excel database, version 2019. The GraphPad prism 8 software was used to carry out statistical analyses of these data. Multiple comparison tests of means were carried out using analysis of variance (ANOVA).

Results:-

Microbiological properties of the different categories of pasteurized RTDjuice were presented in Table 2. Microbiological analyses revealed an absence of total coliforms (TC), anaerobic sulphite-reducing agents (ASR) and Staphylococcus aureus in both RTDjuice. However, the presence of total mesophilic aerobic flora (TMAF), yeasts and moldwere noted in certain combination of temperature and time of pasteurization. Indeed, the microbiological analyses revealed the presence of TMAF at 70°C for 10, 15 and 20 min and at 80°C for 10 in both RTDjuice. On the other hand, for the other combined temperature and time of pasteurisation, we did not find the TMAF. Yeasts and mold were found to be 85 CFU/ml and 97 CFU/ml respectively in PJ and WPJ at 70°C for 10 min, but its content was less than 1 CFU/ml at other treatment parameters.

The results of the pH and Brix degree of both RTDjuices were shown in Table 3. The pH values were found to be significantly high in WPJ (4.17 ± 0.01) as compared to PJ (4.07 ± 0.01) at all treatment parameters. There was not significant effect ($p < 0.05$) due to the time and temperature of the treatment. Soluble dry matter, (Brix degree), which represents the all solids dissolved in water, including sugars, salts, proteins and carboxylic acids (Messaid, 2008), was significantly high in PJ (16.21 ± 0.08) as compared to WPJ (11.4 ± 0.09). The treatment parameter did not significantly affect the soluble dry matter.

The results obtained for vitamin C of bothRTDjuiceswere shown in Figure 1. Vitamin C content in PJ was significantly high (14.70 ± 0.10) than in WPJ (13.50 ± 0.10) at all pasteurization parameters. At 70°C, the amount of vitamin C in both samples did not decrease significantly with increasing time, but at high temperatures the decrease in vitamin C content appeared to depend significantly on processing. Vitamin C content significantly decreased in both samples with increasing processing temperature at all times. In fact, vitamin C content in PJ was observed to be 14.70 ± 0.10 and 9.30 ± 0.20 for 70°C per 10 min and 90°C per 10 min, respectively.

The results of the phytochemical screening were shown in Table 4. The presence of coumarins and reducing sugar was revelated in both RTD juices but alkaloids, flavonoids, triterpenes and saponins were not detected.

The chromatograms obtained after HPLC were presented in Figure 2. The chromatograms showed a peak around 7.2 minutes in both RTD juices and in the herbal tea of Artemesiaannuapurchasedat the pharmacy.

Discussions:-

The total absence of total coliforms and ASR in the RTD juices established an effectiveness of the pasteurization parameters on these groups of germs. However, the total mesophilic aerobic flora, yeasts and mold were observed at certain pasteurization parameters, but their values were well below than those set by the KEBS criteria (10^5CFU/ml for TMAF and 100 CFU/ml for yeasts). It appears from these analyses that all pasteurized RTD juices had a satisfactory microbiological quality in relation to the criteria in force.

The high pH value of WPJ might be associated to the fact that water, which has a pH around 7, was used during its production. However, pH values of both RTD juices were between the interval fixed by the codex which is 3.3 – 5.2 (CODEX STAN 247, 2005). In fact, it was reported that the pH of pineapple juices in different part of Benin was between 3.76 and 4.62(Azonkpin et al. 2019). According to these results, both RTD juices had an acidic pH, so this would promote good preservation of functional foods and constitute an obstacle for microbial proliferation (Sadler and Murphy, 2010). The significant difference between the Brix degree of both RTD juices might be justified by the addition of water intoWPJ. However, the Brix degree of the both juices were comparable to those obtained in Benin,where it ranged between 12.70 and 17.37°Brix (Azonkpin et al. 2019) and were included in the quality standard of the Food Codex which recommends for pineapple juice a Brix degree value between 12.0 and 16.0 (Codex STAN 245,2004). The absence of effect related to the treatment parameter might be associated to the fact that the bottles were hermetically sealed during the heating, which avoids material transfer between the samples and environment (Kaddumukasa et al., 2017).

The high vitamin C content in PJ might be related to the reduction of vitamin due to the use of the water for the production of WPJ. The decrease in vitamin C content due to the process might be associated to the thermolabile behaviour of the vitamin C at high temperature (Rattanathanalerk et al., 2005). However, the presence of vitamin C content, which is a key parameter for assessing of the nutritional quality and potential health benefits from fruit juices (Bull et al., 2004), confirmed that the pasteurization parameters used in this study were efficient. That vitamin C could play an antioxidant activity in these RTD juices (Guenoune et al., 2010).

The presence of coumarins might be associated to its extraction from A. annua leaves (Weathers et al., 2013). It was reported that the mean coumarin in A. annua was scopoletin, which exhibited an antioxidant, hepatoprotective, and anti-inflammatory activities (Malik et al., 2011). That means its presence in the RTD juice might contribute to affect malaria infection (Weathers et al., 2013). The presence of reducing might be related to the use of pineapple as sweetener (Huang et al., 2021). Its presence might improve the acceptance of the juice and may avoid the adding of the sugar into the juice. The absence of alkaloids, flavonoids, triterpenes and saponins might be explained by a variation in the chemical composition of Artemisia annua depending on ecological factors of the environment in which the plant was harvested. These factors have a capital importance in plant syntheses (Mazandarani et al., 2012).

The identification of artemisinin in the RTD juice was done by comparing its retention time using the modified method of Stringham et al. (2018) and the herbal tea as a control. The retention time was analogous to those obtained by Stringham et al. (2018) who found the peak of artemisinin around 7.2 min from precursors of waste streams of Artemisia annua extraction. This confirmed that the peaks observed in the chromatograms might be associated to the presence of artemisinin in the RTD juice. However, that peak was very low, we might hypothesize that theRTD juice contained the artemisinin and it could be used to treat malaria.

Table 1:- Different food microbes enumerated in the RTDpineapple juice.

Germ	**Reference of the method used**	**Reactive culture media**	**Temperature/duration of incubation**
Total aerobicmesophilic (TAM)	NF EN ISO 4833-1	PlateCount Agar	30°C/24 -72hours
Total coliform (TC)	NF V08-050, 1992	VRBL	30°C/24hours
AnaerobicSulphiteReductors (ASR)	NF EN ISO 15213	TypotonesulfiteNeomycine	37°C/24-48hours
Staphylococcus aureus	NF EN ISO 6888-1	Baird Parker	37°C/24-48hours
Yeast and Molds (Y&M)	NF EN ISO 21517-152	Sabourand + Chloramphenicol	30°C/48-72hours

Table 2:- Microbiological analysis of pineapple juice.

	TMA (UFC/ml)	**TC (UFC/ml)**	**Y&M (UFC/ml)**	**ASR (UFC/ml)**	**S. aureus (UFC/ml)**
Criteria of (KEBS)	**1.10^5**	**< 1**	**100**	**<1**	**< 1**
PJ (Pineapple juice)					
70°C-10 min	520	<1	85	<1	<1

70°C-15 min	82	<1	<1	<1	<1
70°C-20 min	4	<1	<1	<1	<1
80°C-10 min	<1	<1	<1	<1	<1
80°C-15 min	<1	<1	<1	<1	<1
80°C-20 min	<1	< 1	< 1	< 1	< 1
90°C-10 min	<1	<1	<1	<1	<1
90°C-15 min	<1	<1	<1	<1	<1
90°C-20 min	<1	<1	<1	<1	<1
WPJ (Water pasteurized juice)					
70°C-10 min	500	<1	97	<1	<1
70°C-15 min	100	<1	<1	<1	<1
70°C-20 min	10	<1	<1	<1	<1
80°C-10 min	4	<1	<1	<1	<1
80°C-15 min	<1	<1	<1	<1	<1
80°C-20 min	<1	< 1	< 1	< 1	< 1
90°C-10 min	<1	<1	<1	<1	<1
90°C-15 min	<1	<1	<1	<1	<1
90°C-20 min	<1	<1	<1	<1	<1

Table 3:- pH and °Brix of the different samples. Different letters indicate significant differences among samples ($P \leq 0.05$). where the small letters indicate the effect of temperature and the big letters indicate the effect of time of the formulation. The letters "a" and "A" were attributed to the highest value.

	pH		**°Brix**	
Pasteurizationparameters	**PJ**	**WPJ**	**PJ**	**WPJ**
70°C-10 min	4.07 ± 0.01aB	4.17 ± 0.01aA	16.10 ± 0.10aA	11.15 ± 0.17aB
70°C-15 min	4.07 ± 0.01aB	4.17 ± 0.01aA	16.30 ± 0.10aA	11.17 ± 0.15aB
70°C-20 min	4.07 ± 0.01aB	4.17 ± 0.01aA	16.27 ± 0.21aA	11.13 ± 0.12aB
80°C-10 min	4.07 ± 0.01aB	4.17 ± 0.01aA	16.20 ± 0.1oaA	11.20 ± 0.12aB
80°C-15 min	4.07 ± 0.01aB	4.17 ± 0.01aA	16.25 ± 0.06aA	11.21 ± 0.06aB
80°C-20 min	4.07 ± 0.01aB	4.17 ± 0.01aA	16.20 ± 0.10aA	11.30 ± 0.06aB
90°C-10 min	4.07 ± 0.01aB	4.17 ± 0.01aA	16.20 ± 0.12aA	11.20 ± 0.17aB
90°C-15 min	4.07 ± 0.01aB	4.17 ± 0.01aA	16.27 ± 0.06aA	11.23 ± 0.16aB
90°C-20 min	4.07 ± 0.01aB	4.17 ± 0.01aA	16.20 ± 0.10aA	11.21 ± 0.23aB

Table 4:- Phytochemical compounds.(-) absence and (+) presence of the compound into the RTD juice.

Compounds	**PJ**	**WPJ**
Alkaloids	-	-
Reducing sugar	+++	++
Coumarin	++	++
Triterpenes	-	-
Tannins	-	-
Flavonoids	-	-
Saponins	-	-

Figure 1: -

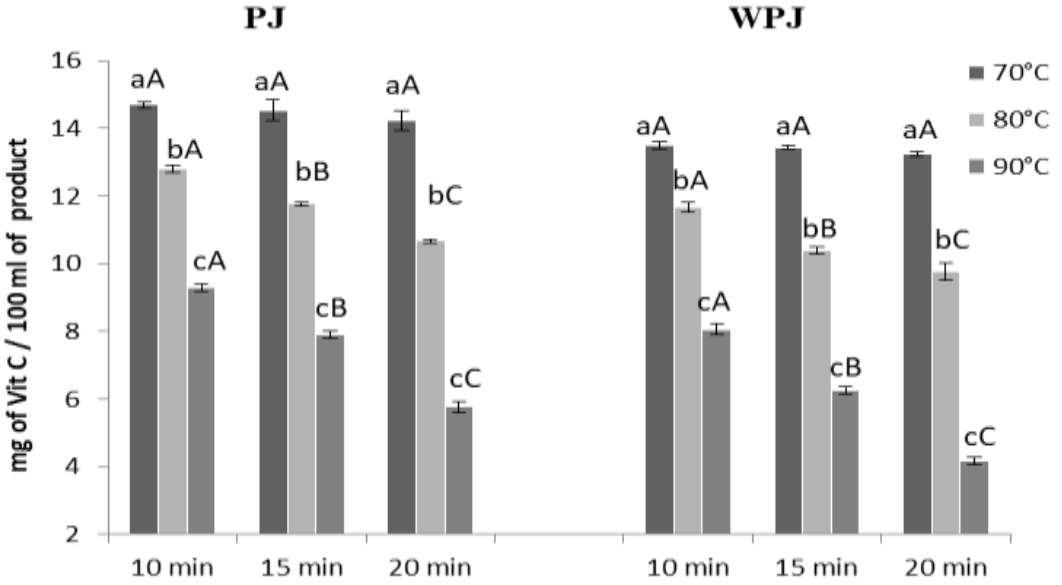

Figure 2:-

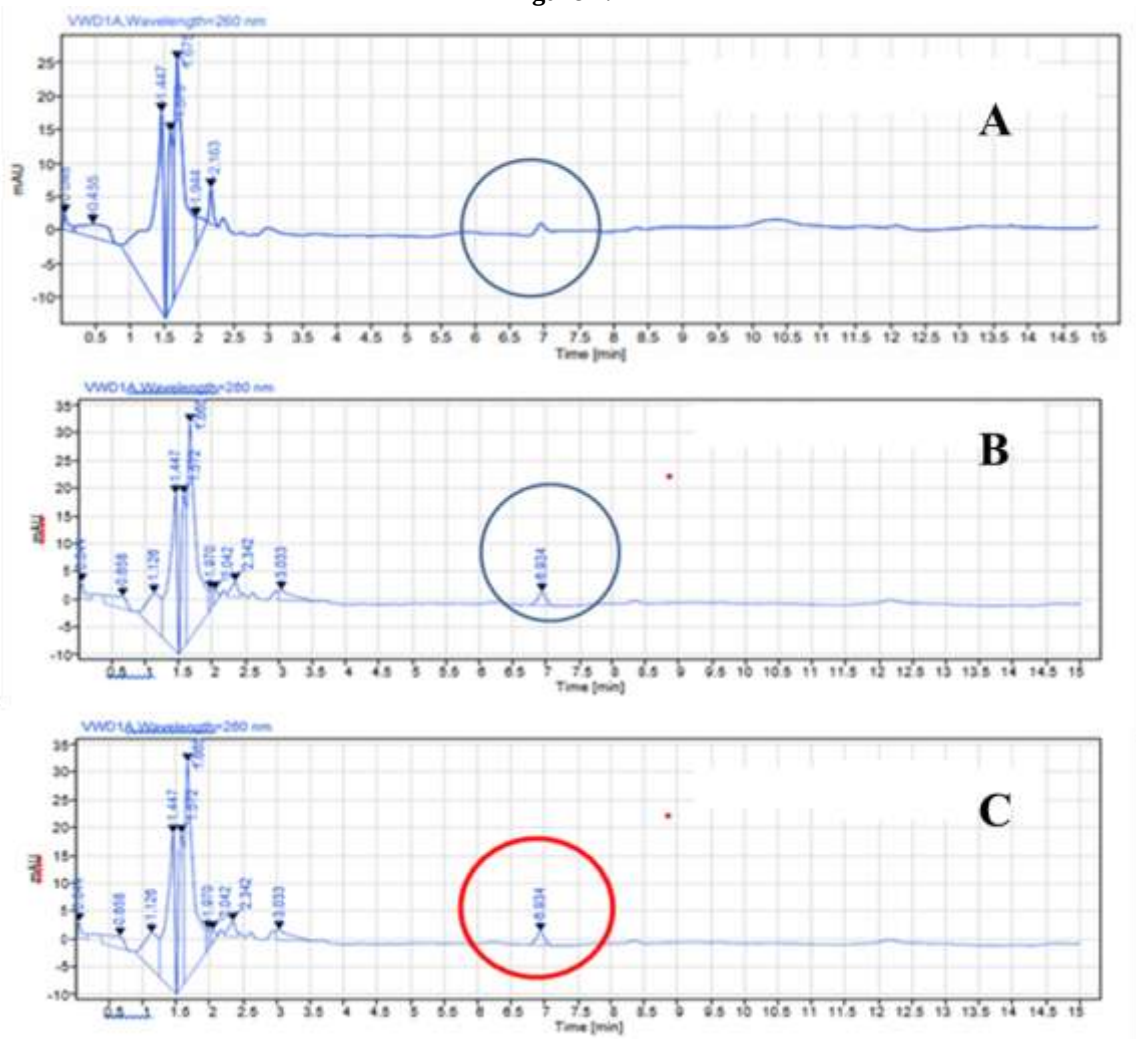

Figure captions

Figure 1

Effect of thermal treatment on the vitamin C concentration. Different letters indicate significant differences among samples ($P \leq 0.05$). where the small letters indicate the effect of temperature and the big letters indicate the effect of time of the treatment. The letters "a"and "A" were attributed to the highest value.

Figure 2

Chromatogram of the samples, where **A** was for Artemesiaannua purchased at the pharmacy, **B** for PJ and **C** for WPJ.

Conclusion:-

The RTDpineapple juiceenriched with Artemisia annua produced in this study had satisfactory microbiological and nutritional quality. The presence of artemisinin in both juices might help its acceptability and the RTD juice could be used daily to prevent malaria. It will be interesting to explore the stability of this artemisinin during storage and its reel effect on the consumer's health.

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