Current Clinical and Medical Education

Received 23 Jun 2025 | Accepted 23 Jul 2025 | Published Online 28 Aug 2025

Published By: Vision Publisher

CCME 3 (8), 1-12

vision publisher Medical Journals

ISSN 2942-0792

Peroxynitrite Scavenging and Singlet Oxygen Scavenging Antioxidant, Antifungal Activity and Investigation of Natural Metabolites of Pineapple (Ananas comosus) Peels Ethanolic Extract Using GC-MS Technique

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Abstract:
The pineapple has been extensively cultivated due to the high value of the product in the market and high production volume. Harvest has a crucial part played by the ripening stage that affects sensory quality and shelf life, particularly the non-climacteric fruit since they do not ripen after picking. The aim of this investigation was to study antioxidant and antifungal activity of pineapple metabolites based on metabolite profiling method. The results of the small molecule study revealed thirteen compounds which include alpha-Farnesene, (3Z,6Z), Palmitic acid-1,2-13C2, Tricin, Feruloylputrescine, n-Decanal, 2,4-dichlorobenzoic acid, Caryophyllene, 1-o-p-coumaroylglycerol, Chrysoeriol-7-O-glucoside, alpha-Myrcene, N,-p-Coumaroyl-N,fer Antioxidants chemical substances have the capability to prevent degenerative diseases by supplying electrons or electrons to free radicals. Fruit peroxide scavenging Fruit chemical such as crude methanolic extract of the uproot fructus; ethyl acetate, ethanol, and gallic acid (standard) expressed were 709.00±25.45, 645.80±20.00, 673.31±22.10 and 812.00±29.07 while recorded 49.00±3.71, 28.50±2.46, 36.09±2.00 and 43.26±2.19 respectively for Singlet oxygen scavenging. When compared to amphotericin B (Am B) and nystatin (NY), the antifungal activity of methanol and ethanol, which are natural metabolites of pineapple (Ananas comosus) peels, were 25.91±0.35, 21.05±0.29, 35.00±0.58 and 31.64±0.53 in *Candida albicans* while recorded 15.40 ±0.24, 17.09±0.25, 24.09±0.33 and 22.00±0.31 in *Cladosporium herbarum*. In the same time recorded 16.70±0.25, 11.98±0.21, 17.00±0.25 and 22.04±0.29 for *Trichophyton rubrum*. Antifungal activity of *Fusarium oxyporum* 15.12±0.24, 20.09±0.27, 21.09±0.28 and 27.00±0.41 while record 29.00±0.43, 23.08±0.32, 37.90±0.59 and 29.04±0.54 in *Cladosporium herbarum.*

Keywords: Peroxynitrite, Singlet Oxygen Scavenging, Antifungal, Metabolites of Pineapple, Peels, GC-MS Technique

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INTRODUCTION

A non-climacteric fruit example is a pineapple. The rate of respiration of climacteric fruit is highest during ripening whereas the non-climactrac fruit has the lowest rate with no pronounced change in respiration. Cymatry is characteristic of climacteric fruit. Other than degreening, ethylene treatment does not affect non climacteric fruit. The quality dependence is critical on non-climacteric fruit harvest being harvested at the right stage of ripening to ensure quality since, other than it being harvested at the right time, such fruit do not further ripen after the harvest. In pines, the leaves that grow above pineapple fruits are known as pineapple crowns used to reproduce vegetatively [1-3]. In the majority of cases, this section of the crown is picked alongside with the fruit. As a pineapple develops, its crown and peel develop. To have a full contribution of the processes of pineapples ripening, one has to examine not only the meat but also the peel and crown [4]. Metabolomics which is an in-depth study of metabolites, and other techniques of tracking changes in metabolites can provide clues to the ripening process in pineapples. Nonetheless, metabolomics has offered little insight into fewer processed fruits such as cherry, blackcurrant, blueberry, non-climacteric melon, and pineapple, and non-climacteric melon fruits. Previously, GC-MS and LC-MS have been used to investigate metabolomics of non-climacteric fruits. To analyze volatile compounds as ripened, various studies used headspace-solid phase microextraction (HS-SPME) in combination with the usage of gas chromatography-mass spectrometry. Research on the ripening of pineapple has focused mainly on volatile and phenolic constituents, and this has been reported by electrospray ionization-mass spectrometry, high-performance liquid chromatography with diode array detection, as well as by high performance liquid chromatography combined with high performance liquid chromatography. The outcomes of these previous studies showed that there were changes in volatile molecules and phenolic patterns such as coumaroyl isocitrate and S-p-coumaryl [5, 6]. Fruits once consumed only leave the peels on, as wastes in the form of agro-industrial produce. Possible disposal (solid-waste management) and environmental consequences of the separation and disposal of the peels of the fruit in the municipal landfills once consumed with edible portion are substantial. Failure to treat such agricultural byproducts has been known to cause huge environmental pollution, odor, soil contamination, and harborage of insects [7]. The environmental concerns are serious considering that when such massive food amounts are put to waste in the landfills, they emit toxic greenhouse gasses. The implementation of agro-industrial waste in the form of converting it into valuable goods is a new form of addressing the waste issue in the environment. Rather than increasing the mounting waste problem, these byproducts of plants can be redesigned into a variety of different compositions, including non-traditional adsorbents, as well as compounds to provide the basis of other chemicals. Besides being a cheap source of obtaining some chemical substances this will solve the issue of solid waste management that on the other hand will contribute less to environmental pollution [8, 9]. Agricultural products are being produced in such large quantities that lignocellulosic biomass is produced, processed and consumed annually in enormous quantities. It can be multiple potential uses of this biomass as cheap bio sorbent, biochemical and biofuel feedstock, enzymes, and metabolites substrates. Also, fruit by-products, such as bagasse, peel, and seeds can be utilized as raw materials to extract the bioactive compounds, minerals, and antimicrobials. Examples of such compounds are phenolics, carotenoids, essential oils, vitamins and minerals. Another option worth pursuing is the utilization of cellulose wastes, as the problems of natural resources depletion and global warming have triggered all the industries to go greener and more environmentally aware. The waste mainly comprises of lot of segments such as the seed, the skin, the rind (peels) the pomace. Such components are full of bioactive compounds that have lots of potential use, such as carotenoids, polyphenols, dietary fibers, vitamins, enzymes, and many others. These waste materials are of much interest to industries because of their biodegradability and the ability to regenerate. Besides being a good solid-waste abating solution, the use of fruit peels has the further advantage of generating income out of garbage [10]. Specifically, the use of fruit by-products, especially the fruit peels that can constitute nearly a third of a fruit in total weight has been precipitated by research that indicated that the peels have more biologically influential characteristics than the other pieces of fruits. This paper set out to assess the antifungal and the antioxidant capacity of an ethanolic extract of the pineapples (Ananas comosus) peels by the use of the GC-MS technique and further visit the natural metabolites.

Materials and Methods

Plant Materials

Preparation of fruit peel

The pineapples were purchased in the city of Hillah in Iraq. Then after washing and letting them dry, we peeled them by using a sharp knife. Then the ingredients were pounded into powdered form and the batch was moved to new container. We employed gas chromatography-mass spectrometry, in order to find out the composition of the oil.

GC-MS Evaluation

The GC-Q/MS analysis was carried out on a GC-MS QP2010 Ultra (Shimadzu, Kyoto, Japan) equipped with InertCap 5 MS/NP column (GL Sciences). Analysis became possible only when the mass spectrometer was adjusted and calibrated. One microliter of the derivatized sample was injected into the split mode injector at an injection temperature of 230 o C with a volume-to-volume ratio of 25:1. The rate by which the carrier gas (He) moved was 1.12 mL/min with linear velocity of 39 cm/s. Two minutes after raising the temperature to 80 ◦C, the column was heated up to 330 ◦C via a heating rate of 15 ◦C / min and held at that temperature within the same time period. The ion source temperature was set at 200 o C and the transfer line was 250 o C. At 0.94 kV, the produced ions were done under electron ionization (EI). Between 85- 500 mass, spectra were taken at 10,000 u / s (check value). To calibrate the analysis of the peak identification, a standard mixture of alkanes (C8-C40) was injected.

Fungal infections and fungal susceptibility in the test-tubes

The pathogenic fungi used in antifungal activity were supplied by Plant Pathology Department. The identified fungal cultures were grown in potato- dextrose- agar (PDA) and any surplus cultures stored below 4 o C. Mycelial plugs (diameters of 2 mm) collected at the edge of each culture were incubated in the middle of each PDA plate (85 mm). In the case of the control plates, DMSO (10%) was also used as opposed to EO. The sample plates were then incubated at 26+/- 2 o C until mycelial plug on the control plate reached the plate edge. The plates were sterilised aseptically The inhibition of growth of each test plate was ascertained relative to the control plate. Experiments were repeated triple times.

Scavenging peroxynitrite

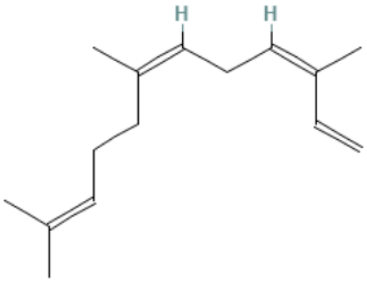
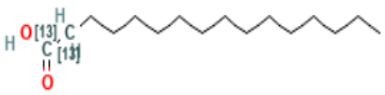
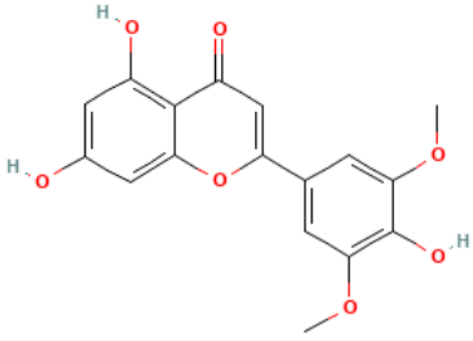
The method was applied in the synthesis of peroxynitrite (ONOO-) [11, 12]. After a short duration being put in an ice bath, 5 milliliters of 0.6 M KNO2 and 5 milliliters of 0.7 M H 2 O 2 were added to 5 milliliters of cold water. 12.2 M NaOH was then added. The excess H 2 O 2 was removed by treating the reaction mixture with granular MnO 2 that was first prewashed with 1. 2 M NaOH. The mixture was left to incubate overnight at -20 o C. The concentration was determined using spectrometry at 302 nm (epsilon = 1670 M -1 cm -1) by drawing the peroxynitrite solution out of the frozen mixture. The scavenging of peroxynitrite was quantified in an Evans Blue bleaching assay In a final volume of 1 ml, the reaction mixture contained 50 mM phosphate buffer (pH 7.4), 0.1 mM DTPA, 90 mM NaCl, 5 mM KCl, 12.5 mM Evans Blue, and the reaction mixture also contained various quantities of the plant extract (0-200 mM) and 1 mM peroxynitrite. The absorbance was read at 611 nm, and allowed to incubate 30 minutes at 25 0 C. The comparison of test and blank sample results have helped us to determine the ratio of ONOO-scavenging. We repeated each test six times.

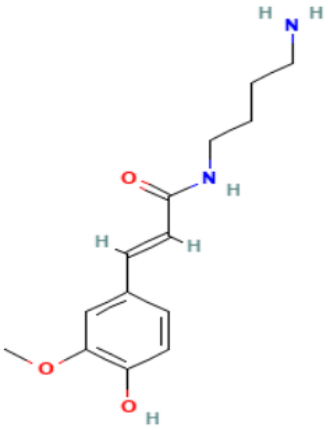
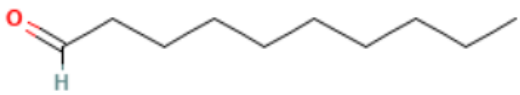
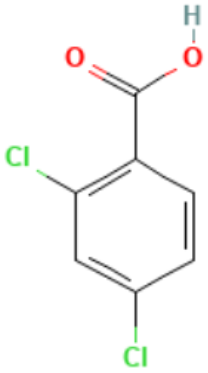
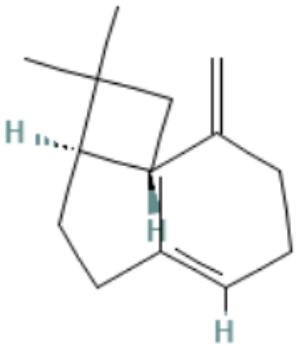
O_2 singlet scavenging

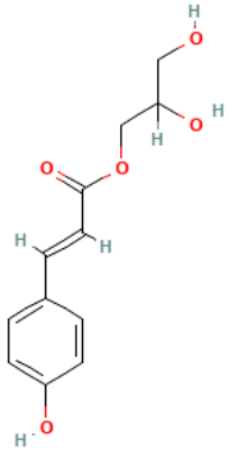
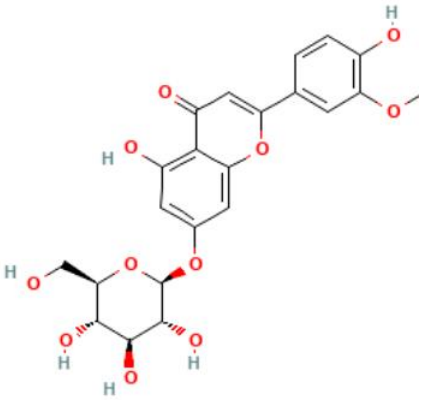
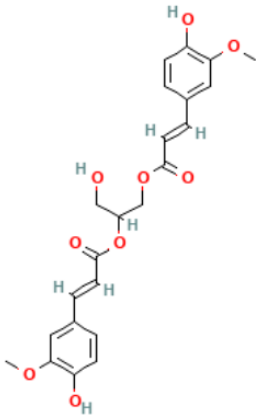
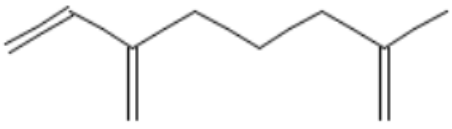
In an earlier mentioned spectrophotometric method, the formation of the singlet oxygen (1O 2) was measured by observing the bleaching of N, N-dimethyl-4-nitrosoaniline (RNO). The singlet oxygen is obtained by reacting NaOCl and H2O2 and there was a bleaching of RNO observed at 440 nm. The reaction mixtures contained 45 mM phosphate buffer (pH 7.1), 50 mM NaOCl, 50 mM H 2O 2 50 mM histidine, 10 mM RNO and various concentrations samples (0-200 mug/ml) in a final volume of 2 ml. The decrease in RNO was determined at 440 nm in 40 min at 30 0 C. The scavenging activity of the scavenge was tested against a reference chemical which is lipoic acid. Each experiment was done six times [13].

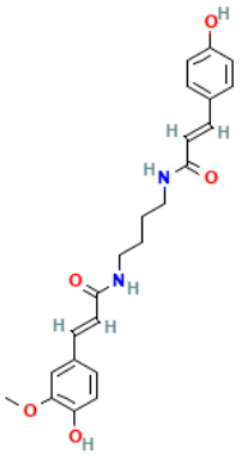
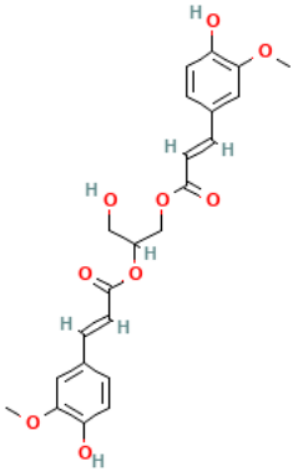
RESULTS and DISCUSSION

The findings were 13 compounds namely, alpha-Farnesene, (3Z,6Z), Palmitic acid-1,2- 13C2, Tricin, Feruloylputrescine, n-Decanal, 2,4-dichlorobenzoic acid, Caryophyllene, 1-o-p- coumaroylglycerol, Chrysoeriol-7-O-glucoside, alpha-Myrcene, N, -p- Coumaroyl-N- feruloyl It is also established that metabolic profiling is the most helpful method of examining a substantial range of metabolites that are each represented by a unique set of chemicals that obviously respond dynamically to factors in the environment or to physiological stimuli which can impact on development. This study pertaining to the pineapple ripening process utilised a GC-MS based profiling of metabolites. The sensitivity, repeatability and the capability to quantify many metabolites in a single extraction step make GC-MS a perfect metabolite profiling method [14, 15]. Metabolite in pineapple was identified to be a wide variety of chemicals, which included sugars, amino acids, amines, etc., organic acids. Findings have revealed that pineapple contains high quantities of sugars. This agrees with previous studies that concluded the samples of pineapple flesh had a lot of concentrations of sugar. To study the annotated metabolites of each supplement I used PCA and OPLS. Principal component analysis (PCA), a form of multivariate data analysis, may be able to uncover sample variance using metabolites as an explanatory variable.

No.	Compounds	Structure	Molecular Formula	Molecular Weight
1.	alpha-Farnesene, (3Z,6Z)		$C_{15}H_{24}$	204.35 g/mol
2.	Palmitic acid-1,2-13C2		$C_{16}H_{32}O_2$	258.41 g/mol
3.	Tricin		$C_{17}H_{14}O_7$	330.29 g/mol

4.	Feruloylputrescine		$C_{14}H_{20}N_2O_3$	264.32 g/mol
5.	n-Decanal		$C_{10}H_{20}O$	156.26 g/mol
6.	2,4-dichlorobenzoic acid		$C_7H_4Cl_2O_2$	191.01 g/mol
7.	Caryophyllene		$C_{15}H_{24}$	204.35 g/mol

8.	1-o-p-coumaroylglycerol		$C_{12}H_{14}O_5$	238.24 g/mol
9.	Chrysoeriol-7-O-glucoside		$C_{22}H_{22}O_{11}$	462.4 g/mol
10.	1,2-Di-O-feruloylglycero		$C_{23}H_{24}O_9$	444.4 g/mol
11.	alpha-Myrcene		$C_{10}H_{16}$	136.23 g/mol

12.	N,-p-Coumaroyl-N'-feruloylputrescine		$C_{23}H_{26}N_2O_5$	410.5 g/mol
13.	1,2-O-Di-trans-feruloylglycerol		$C_{23}H_{24}O_9$	444.4 g/mol

Upon using GC-MS, the chemical constituents of the oils which were retrieved in the pineapple peel was limonene, palmitic acid, alpha-farnesene, trans caryophyllene, myrcene, and 1-cyclohexene- 1-carboxaldehyde. The content of a limonene was elevated, being higher than 75 percent, and could be considered as the main constituent of the oil. Limonene has a large variety of uses in the cosmetics industries and the health industry as an ingredient in fragrances and dietary supplements. It also enters into food production and the efforts to manufacture many medicines. To identify the metabolite that is associated to the ripening phases use the variable significance in projection (VIP) scores to indicate the contributing metabolites. Based on these scores, the five most important metabolites in the meat include - melezitose, inositol, xylonic acid, gluconic acid, and raffinose; in the peel, they include - inositol, mannose, galactose, sucrose and aspartic acid [16]. These results demonstrated that these metabolites were either increased or reduced upon the ripening process of the pineapples. In addition, inositol could be oxidized to D-glucuronic acid, a major component of cell wall of the plant Arabidopsis. Therefore, the presence of inositol in fruit can also be linked to cell wall. It has been discovered that melezitose is connected with the system of osmoregulation. However, the relative intensity pattern when compared with inositol, the osmoregulation mechanism might be regulated differently during the ripening of pineapple by these two metabolites. Melezitose has a poor relative intensity when compared to other sugars, but in a previous study found that it does attract ants, in particular in honeydew fruit. Consequently, there is the possibility that the beauty of the fruit at optimal ripening will be reflected in the accumulation of the melezitose in the late ripening stages. Certain fruit alterations such as development of a change in skin color or softness, could be a consequence of an increase in the presence of oxidative stress during the ripening process. The ascorbic acid, an antioxidant is a well recognized fact to be present in fruits [17]. As explained in the late article, xylonic acid is formed when ascorbic acid is broken down and that is why it increments as the fruit ripens off. Studies on the pineapples indicate there is a decrease in raffinose during the ripening of the fruit. In previous study, this observation was confirmed, through declining raffinose levels with maturity in a non-climacteric cultivar of Japanese plum. Therefore,

the ripening might be through reduction-oxidation mechanism which is linked to xylonic acid and the amount of raffinose. In the late stages when pineapples ripen, researchers observed an increase in gluconic acid concentrates they contained. A rise in the availability of carbon molecules in the later stages of pineapple ripening could be the reason behind its rise. Because of the exogenous degradation of the cells, altered composition of the cuticles, and pH outcomes of cell host, fungi might enter into the aggressive colonization phase that, in turn, enhances the intensity of gluconic acid. In vegetables such as mangoes and apples, the reduction in the pH in the form of gluconic acid may be indicative of an infection that produces this acid. However, the amount of sucrose and galactose in the skin of pineapples could vary with fruit maturity, as is shown in this trial adding more metabolites to those already published. Sucrose was a well-known sweetening agent and sweetener of choice in most recipes [18, 19]. That being said, the fructose in the peel is likely not to have a direct influence on sweetness levels of the flesh itself. There seems to be some effect that the sucrose content of the peel is low as compared to the flesh. Strawberries are not climacteric fruits, but sucrose aids in controlling their development and ripening as well as lending sweetness. The correlation could be the attachment of galactose to plant cell wall. Prior studies have shown that as fruit ripens, the level of galactose, which is the main non-cellulosic sugar in the cell wall, drops off greatly. The classes of metabolites with high VIP scores are organic acids and sugars including galactose, melezitose, inositol, raffinose and sucrose. There are numerous biological processes that occur during ripening such as cell walls relax, texture develops, flavor is formed, and the chlorophyll is broken down and the pigment is deposited [20-23]. The ripening of pineapple might be associated with alterations in the texture as evidenced by alternations in the levels of melezitose and inositol. Mezelitose and inositol are known to dictate the texture and the hardness of fruits. Also, the levels of inositol, galactose, mannose, and allowing the cell wall to loosen during the ripening process may be correlated. The three main sugars that occur in plant cell walls are the inositol, which is precursor to the D-glucuronic acid, the galactose which is a primary non-cellulosic sugar and the mannose which appears in the hemicellulose. In this way a relationship between the reduction of these three metabolites and a cell wall thinning, usually linked to a fall of firmness and an increase of gluconic acid, could be observed. Besides the regulation of the programmed cell death and the aging of a cell, reactive oxygen species is involved in the process of the fruit ripening. As stated previously, both of the metabolites were known to interreact with reactive oxygen species. Certain biochemical changes that are involved in the ripening process execute their effects through plant hormones in the various biological systems. Aspartic acid and sucrose change during ripening, and could affect auxin and abscisic acid respectively [24]. Therefore, it may control pineapples ripening.

Figure 1. Peroxynitrite scavenging of fruit Crude methanolic extract, ethyl acetate, ethanol fractions and Gallic acid (standard) of Pineapple (*Ananas comosus*) Peels

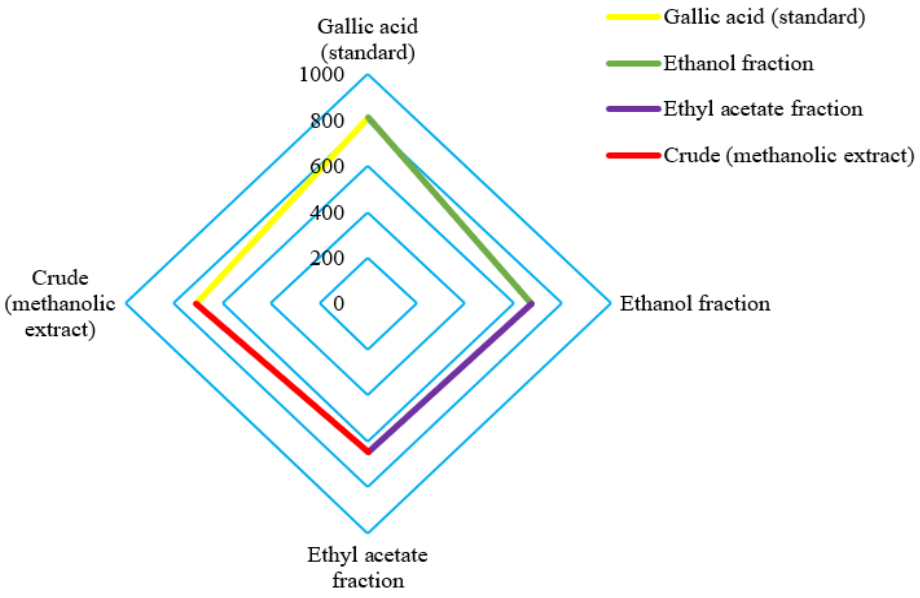


Figure 2. Singlet oxygen scavenging of fruit Crude methanolic extract, ethyl acetate, ethanol fractions and Gallic acid (standard) of Pineapple (*Ananas comosus*) Peels

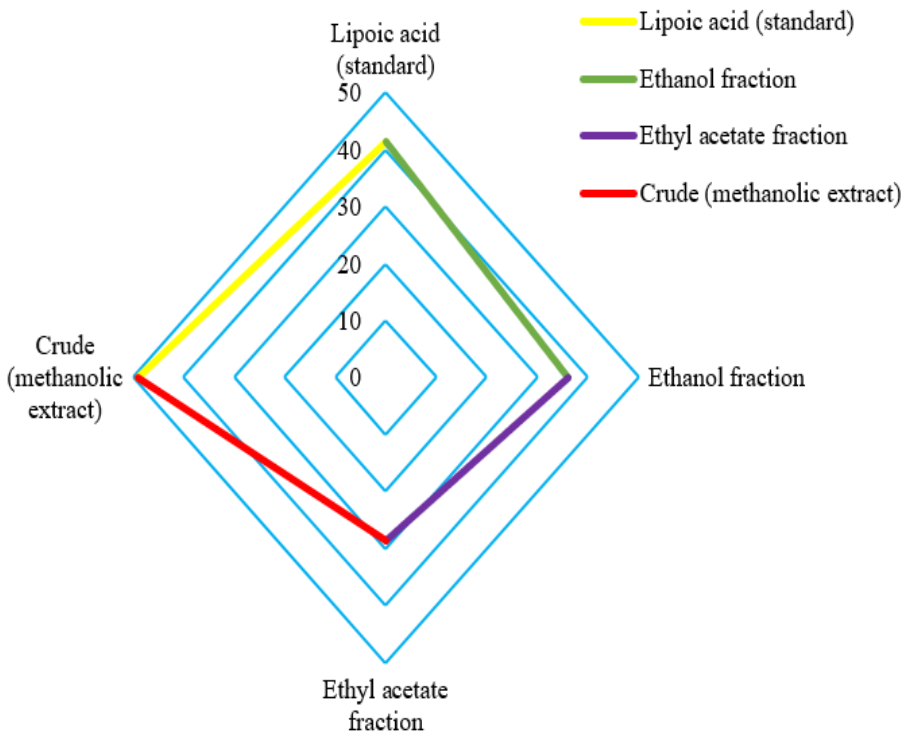


Figure 3. Antifungal activity of natural metabolites of Pineapple (*Ananas comosus*) Peels against *Candida albicans*

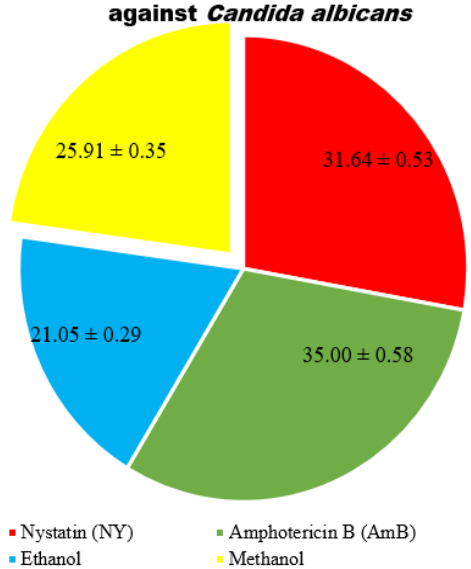


Figure 4. Antifungal activity of natural metabolites of Pineapple (*Ananas comosus*) Peels against *Cladosporium herbarum*

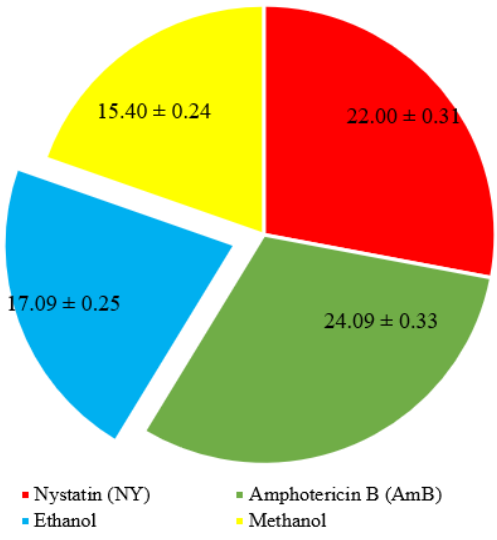


Figure 5. Antifungal activity of natural metabolites of Pineapple (*Ananas comosus*) Peels against *Trichophyton rubrum*

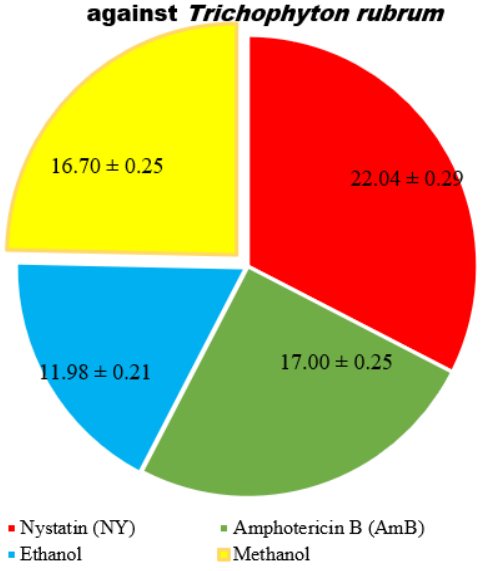


Figure 6. Antifungal activity of natural metabolites of Pineapple (*Ananas comosus*) Peels against *Fusarium oxyporum*

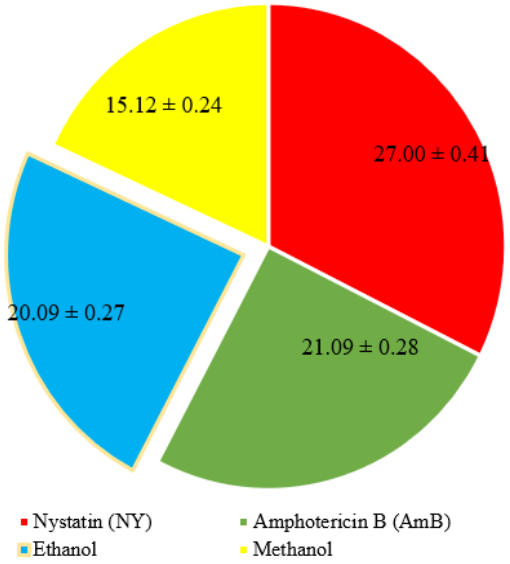
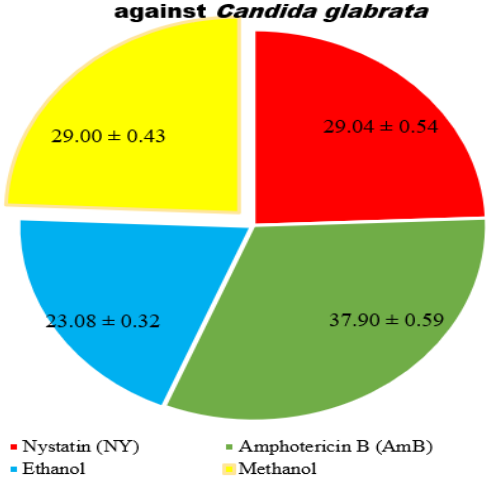


Figure 7. Antifungal activity of natural metabolites of Pineapple (*Ananas comosus*) Peels against *Candida glabrata*



Singlet oxygen Scavenging and fruit peroxide scavenging Pineapple (Ananas comosus) peel chemicals including crude methanolic extract, ethyl acetate, ethanol fraction and gallic acid (Standard) showed 709.00±25.45, 645.80±20.00, 673.31±22.10 and 812.00±29.07 while recorded 49.00±3.71, 28.50±2.46, 36.09±2.00 and 43.26±2.19 respectively for Singlet oxygen scavenging.

Pineapple is harshly slain by factory processing That said, there is an abundance of nutritionally unavailable anti-oxidant rich and aromortically variable nonedible waste that the fruit generates in vast amounts. The presence of these features shows that these waste products can be reused as an agri-food waste in pharmaceutical, medicine, and even the food industry. The Total Phenolic Content of a fruit byproduct is one mechanism of measuring its antioxidant capacity. Many factors, such as the variety of fruits, their ripeness, agronomical growing conditions, etc., define the profile and content of phenolic compounds. Because the antioxidant and phenolic chemicals require extraction preceding their determination, the solubility of the solvent employed will also impact recovery of the goods. Colorimetric methods easily used include Folin-Ciocalteu method and very easy to follow when comparing sample processes that are used in the same experimental conditions. The approach is founded on such techniques, that is why it is widely used. Relative to amphotericin B (AmB) and nystatin (NY), methanol and ethanol which are natural constituents of pineapple (Ananas comosus) peels recorded 25.91+/-0.35, 21.05+/-0.29, 35.00+/-0.58 and 31.64+/-

0.53 in Candida albicans and 15.40 +/-0.24, 17. In the same time took 16.70+/-0.25, 11.98+/-0.21, 17.00+/-0.25 and 22.04+/-0.29 against Trichophyton rubrum. The fungicidal activity of Fusarium oxyporum 15.12±(0.24) 20.09±(0.27) 21.09±(0.28) and 27.00±(0.41) whereas record 29.00±(0.43) 23.08±(0.32) 37.90±(0.59) and 29.04±(0.54) in Cladosporium herbarum (Figure 3-7). Generally, ethyl acetate content was high in pineapple peel sample compared to the core sample, isopentyl acetate was high in the core sample as well. Moreover, the concentrations of ethyl acetate in all the samples differ significantly which might be directly linked to the difference in the chemical composition, environmental development conditions, pineapple variety, the stage of maturity, and other characteristics. Ethyl acetate found also in pineapple samples is formed when higher alcohols including isoamyl are combined with acetyl CoA that is formed following the breakdown of carbs or amino acids, as mentioned earlier. In such a way, the ultimate quantified concentration of ethyl acetate directly relates to the mixture of pineapple by-product. The reason is that the peel contains much cellulose hemicellulose, lignin, waxes, pectin and ash [27, 28]. Due to increased yields of alcohols in the degradation of carbohydrates, it is plausible that the higher level of ethyl acetate in pineapple peel samples may be attributed to a greater concentration of carbohydrates.

CONCLUSION

We relied on GC-MS to determine the chemical compositions of pineapple peels. It has been established that the following components are present n-decanal, palmitic acid, alpha-farnesene, trans caryophyllene, myrcene, limonene. You may include these compounds to your meal and drink to make it even tastier. This is also the case of limonene and isopentyl acetate, which could also be found in pineapple core along with other volatiles. In order to harness pineapple extract as an antioxidant and flavoring agent in the food industry, there is need to conduct more studies in the coming years.

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