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Phyto-Constituents, GC-MS Analysis and Antifungal Activity of Methanol Extract of Sweet Orange Peels

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Abstract

Sweet orange peels have been considered waste materials by most people, however recent scientific studies have revealed adequate potentials to include textile manufacturing, mosquitoes-repellant finish, print transfer medium, antimicrobial agents, water purification, bio adsorbent, bio char and biogas among others. This research is aimed at isolating the oil fraction, screening the phyto constituens and determining the antifungal activity of methanol extract of sweet orange peel. The orange peels were isolated from the orange, grated into pieces and extracted using methanol solvent by soxhlet extractor. The crude extract was subjected to phytochemical screening and antifungal activity while vacuum liquid chromatography was used to isolate the oil from the peel extract and characterized by gas chromatography mass spectrometry (GC-MS) Saponins, alkaloids, tannins, phenolics and flavonoids were present while zone of inhibition of the methanol extract against Candida albican Penicillium notatum and Epidermophyton floccosum gave 15 mm, 10 mm and 17 mm at 100 mg/mL dose respectively. The major component of the oil were 17-octadecynoic acid (Retention time (Rt):15.469, 36.04%) a saturated fatty acid, 8 methyl-6-nonenoic acid (Rt: 10.84, 10.4%) an unsaturated fatty acid and pentadecanoic acid (Rt: 14.286, 9.34%) a saturated fatty acid. This research thus confirmed the presence of bio active chemical constituents with potentials for inhibiting the growth of some fungi infections.

1.0. Introduction

The role of medicinal plants in health care management in rural areas of Nigeria is enormous and these plants and their products are gifts to mankind from nature. Many of the chemical constituents derived from their parts have various physiological potentials among which are anxiolytic [1] and [2], antimalarial [3]and [4], antimicrobial [5], aphrodisiac, antiviral [6], uterine contractile [7], antiasthmatic [8] and anti-cancer activity [9] among others. Sweet orange (*Citrus sinensis*), family-*Rutaceae* is the most common fruit tree cultivated by man all over the world especially in most African habitats [10]. In English, it is called sweet orange and locally referred as: “Alimoi” (Edo), “Leemun” (Hausa) and “Osan” (Yoruba). The tree can grow up to 6 meters (20 feet) in height and the broad, glossy, evergreen leaves are medium–sized and ovate while the petioles (leafstalks) have narrow wings [10]. Essentiasl oil from sweet orange is a byproduct of the juice industry produced

by pressing the peel and it is applied as a flavoring of food, drinks and as fragrance in perfume and aromatherapy [11] The bioactive chemical constituents of the essential oil in orange peel among others include lirnonene, geranial, alpha pinene, citronellial, sabinene, myrcene and neral. Meanwhile, for appropriate classification, the essential oil from the peel is a mixture of over a hundred compounds that can be grouped into three fractions: hydrocarbons, terpenes/terpenoids and oxygenated nonvolatile compounds. The terpene fraction can constitute about 90% of the oil [12] D-limonene gives the orange fruit and many other citrus fruit their specificr aroma. It an effective, medicinal and environmentally friendly relatively safe solvent, which makes it an active ingredient of choice in many applications as antimicrobial, drugs, adhesive, stain removers and cleaners of various sorts strippers [12] and [13]. In many countries, orange juice is an essential entity in meals to cater for the everyday recommended dose of vitamin C. The fruits are the richest source of vitamins, minerals, and energy, which has the potential to improve wellbeing4. The regular consumption of orange helps in the effective functioning of the heart and kidney. The fruit also give benefit to the skin, teeth, and bone, along with maintaining the normal blood pressure and cholesterol levels in the body [14]. Orange peel is considered as by-products of the fruit and studies show that they are good sources of bioactive compounds amidst industrial materials[15]. Citrus species have been reported as a source of botanical insecticides while the peel and seed solvent extracts from a variety of citrus plants containing secondary metabolites have been cited to show insecticidal activity against several insect species [16], [17] and [18] .Thus, the identification of toxic secondary metabolites constituents could be the first step in the investigation of natural insecticides based on peel waste. Aishwariya [19], have discovered that orange peels can be re-used as textile materials, bio materials and manure for agricultural productivity. In 2012, Adriana Santanocito in [19], also make fabrics from 100% orange peel waste fibre. Recent studies indicated the re use of orange peel as dyes [20], mosquito repellant [21], water purification [22], biogas [23] and as bio-adsorbent [24]. Report showed that orange peel has been fed to cattle, where it drastically reduce the existence of pathogenic bacteria, such as *E. coli* and S*almonella*, in the intestine of cattle [21]. This antibacterial potency can be possible due to the useful bio active chemical constituents in the orange peels which are considered waste materials. Thus, this study is aimed at determining the phyto-constituents and antifungal activity of the methanol extract of sweet orange peel

2.0. Materials and Methods

2.1. Collection of Plant Samples

The orange fruit were purchased at the Uselu Market in Egor Local Government Area, Benin City, Edo state, Nigeria. The plant fruit samples were identified by Prof. J. F. Bamidele, a Taxonomist in the Department of Plant Biology and Biotechnology, University of Benin, Benin city, Nigeria. The sweet orange peels were obtained from the epicarp with the aid of a grater into tiny pieces. The chopped orange peels were further pulverized into slurry form in preparation for extraction.

2.2. Extraction

Four hundred and seventeen grammes (417g) of pulverized orange peels were extracted with methanol solvent (BDH, England) in a soxhlet apparatus for eight hours. The solvent from extract was removed under reduced pressure and controlled temperature (50 ºC) in a rotary evaporator (RE, 200) at 50°C to obtain a crude sy rupy extract.

2.3. Phytochemical screening of methanol extract of sweet orange peel

The phytochemical screening of the methanol extract of sweet orange peels were performed using standard procedures prescribed by Sofowora, [25]; Trease and Evans [26] and Rajasudha and Manikandan [27].

2.3.1. Test for glycosides (Modified Borntrager's Test)

1 mL of extract was treated were dissolved in 1 mL of ferric chloride solution and immersed in boiling water for 5 minutes. Both mixtures were cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink color in the ammonical layer indicates the presence of anthranol glycosides.

2.3.2. Test for saponins (Froth Test)

1 mL of extract was diluted with distilled water to 20 mL and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.

2.3.3. Test for flavonoid (Lead acetate Test)

2 mL of extract was treated with few drops of lead acetate solution respectively. Formation of yellow colour precipitate indicates the presence of flavonoids**.**

2.3.4. Test for phenolic compounds (Ferric Chloride Test)

1 mL of extract was treated with 3-4 drops of ferric chloride solution. Formation of a bluish black colour indicates the presence of phenols

2.3.5. Test for tannins (Gelatin Test)

To 2 mL of extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.

2.3.6. Test for Eugenols

2 mL extract was mixed with 5% KOH solution. The aqueous layers were separated and filtered. Few drops of dilute HCl were added to the filtrate. A pale yellow precipitate is indicative of a positive test

2.3.7. Test for phytosterols. (Libermann Burchard's test)

0.5 g of extract was treated with 2mL chloroform and filtered. The filtrates were respectively treated with 2 mL of acetic anhydride, boiled and cooled. 2 mL Conc. Sulphuric acid was added to both solution. Formation of a brown ring at the junction indicates the presence of phytosterols.

2.3.8. Test for terpenoids (Salkowski test)

5 mL of extract was mixed in 2 mL of chloroform and 3mls of conc. H_2SO_4 was carefully added down the side of the inner wall of the test tubes to form a layer. A reddish brown colouration of the inter-phase is required for the presence of terpenoids.

2.3.9. Test for Triterpenes (Salkowski's Test)

5 mL of extract was mixed in 2 mL of chloroform and filtered. The filtrates were treated with 3mL of Conc. Sulphuric acid, shaken and allowed to stand. The appearance of golden yellow colour indicates the presence of triterpenes.

2.3.10. Test of Diterpenes (Copper acetate Test)

5 mL of extract was dissolved in 5 mL of distilled water and treated with 3-4 drops of copper acetate solution. Formation of emerald green color indicates the presence of diterpenes

2.3.11. Test for alkaloids (Hager's Test: Hager's reagent (Saturated picric acid solution)

2 mL of Picric acid was added to sweet orange peels extract. A yellowish precipitate test is a positive test.

2.4. Antifungal activity

2.4.1. Microorganisms

The microorganisms employed in this study were procured from the University of Benin Teaching Hospital, Benin City which includes clinical isolates of *Candida albican, Penicillium notatum* and *Epidermophyton floccosum*

2.4.2. Media

Nutrient broth and potatoe dextrose, all products of Himedia Laboratories Mumbai (India) were used in this study. The composition of the medium was Beef extract -3.0 g, peptone - 5.0 g, sodium chloride -8.0 g, agar-15.0 g.

2.4.3. Agar well diffusion assay

The antifungal activity of the methanol extract of orange peel was determined by using agar well diffusion technique. Nutrient agar plates were seeded with 0.1 ml of an overnight culture of each microbe (10^6 CFU/mL). The 24 h broth culture of each fungi was used to seed molten nutrient agar at 45°C, allowed to set and a well was made by sterile standard cork borer (6.0 mm in diameter and 200 µl (0.2 ml) of various concentration of essential oil extract added into each well. The fungi and plates respectively were incubated at 37°C for 24 h after which diameter of zones of inhibition was measured [28].

2.5. Determination of minimum inhibitory concentration

Minimum Inhibitory Concentration (MIC) is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. The MIC values of the methanol orange peel extract was determined using increasing volumes of 12.5 ,25, 50 and 100 mg/mL concentrations and a drop of the fungi suspension that had been previously diluted to 10^6 CFU/ml were aseptically incorporated into molten potatoe dextrose agar and allowed to set. The plates were incubated to at 37°C for 24 hours. The lowest concentration preventing visible growth for each of the test organisms was recorded as the MIC. Ketoconazole was used as the positive control for the fungi. At various concentrations of the extract, the zone of inhibition were measured using a meter rule whose values were then converted to millimeters (mm)

2.5.1. Isolation of oil

Fifty grams (40 g) of the crude methanol extract of the sweet orange peels were partitioned with 250 mL of methanol; distilled water and hexane mixture (ratio: 1:1:2) and shaken vigorously in a separatory funnel. The upper organic fraction was separated, concentrated and then subjected to vacuum liquid chromatography (VLC), using silica gel (particle size: 200-425 mesh) as the solid phase and methanol and hexane mixture ratio (1:1) was used as the mobile phase. A pale yellow oily phase obtained was dried over Na_2SO_4 and concentrated to recover the oil fraction.

2.5.2. GC-MS analysis

The GC-MS analysis of the methanol extract of sweet orange peel was done on a Shimadzu, GCMS-QP2010SE. Separation of the oil was carried out on a HP-5 MS (5% phenylsiloxane) column with nitrogen as the carrier gas with a flow rate, 1.80 ml/min.. The oven programme was set at a temperature of 70 ^{o}C and held for 2 minutes, then it was ramped at a rate of 10^{o}C/min to 280^{o}C and held for 7 minutes

3.0. Results and Discussion.

3.1. Phytochemical constituents

The phytochemical constituents detected in the methanol extract of orange peels is shown in Table 1

Table 1: Phytochemical constituents in the methanol extract of orange peels

S/N	Phytochemical constituents	Methanol extract (*Orange peel*)
1	Glycoside	+
2	Saponin	+
3	Flavonoid	+
4	Phenolics	+
5	Tannin	+
6	Eugenol	+
7	Phytosterols	+
8	Terpenoids	+

9	Triterpenes	-
10	Diterpenes	-
11	Alkaloids	+

Key: - = absent , + = present

Phyto constituents (Table 1) present in methanol of orange peels extracts were saponins, phenolics, alkaloids, phytosterols, terpenoids excluding triterpenes and diterpenes. Tannins, terpenoids and flavonoids were reportedt from the result obtained by Gotmare and Gade in [29] but the water extracts from their work also indicated more phyto constituents including tannins, terpenoids, saponins and flavonoids. These differences can be as a result of choice of solvent used. Alkaloids, flavonoids and saponins were also reported by Kaur et al. [30] while saponins, flavonoids and tannins were present along with glycosides and reducing sugar [31].

3.2. Antifungal Activity

The antifungal activity of the oil fraction extracted from sweet orange peel is shown in Table 2.

Table 2 Antibacterial activity of limonene against some bacteria

Microorganisms	Volumes of control and limonene for Minimum inhibitory concentration (MIC)				
	12.5 mg/mL	25 mg/mL	50 mg/mL	100 mg/mL	Ketoconazole 0.01mg/mL
	Zone of inhibition (mm)				
C. albican	08	08	10	15	30
P. notatum	05	05	07	10	26
E. floccosum	05	10	14	17	29

(–) = no activity, < 10mm = non-significant activity. 10—19 mm = significant activity,

> 20mm = high activity [10].

The antifungal activity of the methanol extract of orange peel (Table 2) indicated zone of inhibition against *C. albican* (15 mm), *P. notatum* (10 mm) and *E. floccosum* (17mm) at 100 mg/mL dose respectively. These results showed a significant activity when compared to the standard antifungal positive control drug, ketoconazole. This results are in agreements with the findings of Agaas [21], whose Findings gave a significant activity with methanol extracts of orange peel for *S. aureus, E. coli, Samonella typhi* and *candidas abican*. More so, the report of Kaur et al. [30] indicated significant activity against selected pathogens with the use of methanol as solvent rather than water. It has also been reported that orange peel extracts when fortified into creams and body lotion can act as antiseptic. In fact, study quoted that the extracts from citrus (lemon and orange) peel waste combined with sodium bicarbonate, coated on a cotton fabric resulted in appreciable anti-odour and anti-microbial properties on the fabric [19].

3.3. GC-MS Analysis

The GC-MS chromatogram of oil fraction of sweet orange peels in Figure 1 showed 13 peaks indicating from the search list of the chemical abstract services thirteen compounds. The chemical compound identified in the oil sample are presented in Table 3.

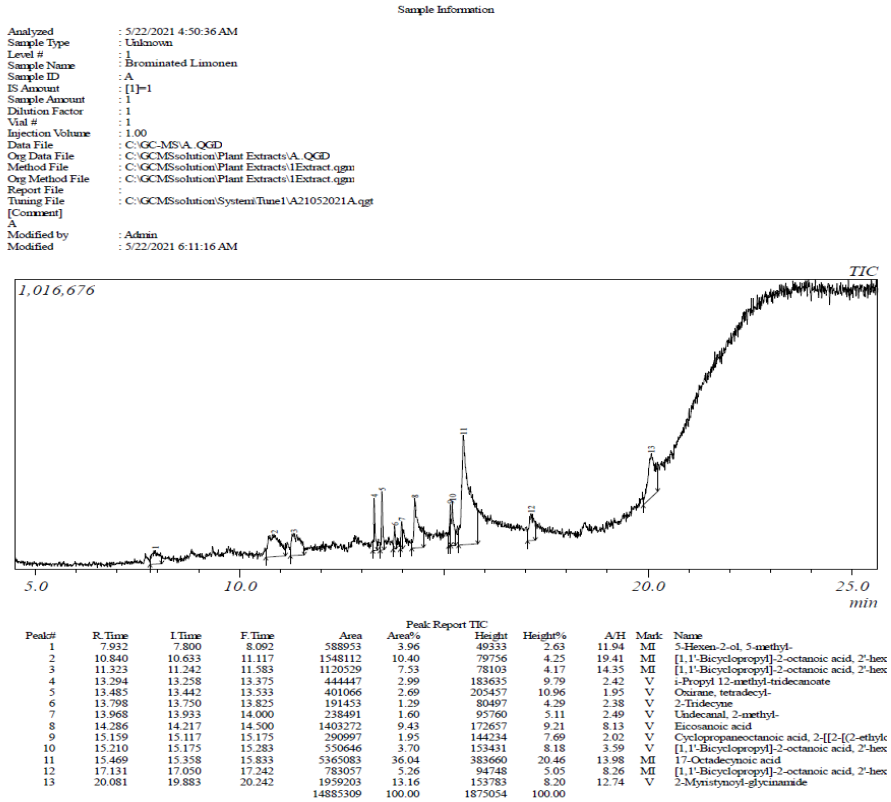

Figure 1: GC-MS spectrum of methanol oil extract of orange peels

Table 3: GC-MS analysis of oil fraction isolated from orange peel.

Peak no	**Retention time (RT, minutes)**	**Name of compounds**	**Area percent %**	**Mol formula**
1	7.932	5-methyl-5-hexen-2-ol	3.96	$C_7H_{14}O$
2	10.840	8-methyl-6 nonenoic acid	10.40	$C_{10}H_{18}O_2$
3	11.323	10-undecynoic acid	7.53	$C_{11}H_{18}O_2$
4	13.294	Tetradecanoicacid,1-methylethyl ester	2.99	$C_{17}H_{34}O_2$
5	13.485	Tetramethyl-2-hexadecan-1-ol	2.69	$C_{20}H_{40}O$
6	13.798	2-tridecyne	1.29	$C_{13}H_{24}$
7	13.968	Hexadecanal-2-methyl	1.60	$C_{17}H_{34}O$
8	14.286	Pentadecanoic acid	9.34	$C_{15}H_{30}O_2$
9	15.159	8-nonynoic acid	1.95	$C_9H_{14}O_2$
10	15.210	17-octadecynoic acid	3.70	$C_{18}H_{32}O_2$
11	15.469	17-Octadecynoic acid	36.04	$C_{18}H_{32}O_2$
12	17.131	2-nonenoic acid	5.26	$C_{16}H_{16}O_2$
13	20.081	N-(2-Amino-2-oxoethyl)-2-tetradecynamide	13.16	$C_{16}H_{28}N_2O_2$

From Table 3, the major component of the oil were, 17-octadecynoic acid (Retention time (Rt):15.469, 36.04%) a saturated fatty acid, 8 methyl-6-nonenoic acid (Rt: 10.84 , 10.4%) an unsaturated fatty acid and pentadecanoic acid (Rt: 14.286,9.34%) a saturated fatty acid. While minor component among others were hexadecanal-2- methyl (Rt 13.968, 1.60%) a saturated fatty

acid and -2-tridecyne (RT 13.798, 1.29%) an unsaturated fatty acid among others. Comparing with the work of Cholke et al. [32], fifteen peaks were detected from the orange peel oil extract by steam distillation method while for this study only thirteen peaks were recorded for the oil fraction using soxhlet method of extraction. This shows that steam distillation method can extract more oil constituents from the oil peel. Cyclohexanol (Rt, 13.480) was detected from the work of Cholke et al. [32] while in this study, a derivative of cyclohexanol, 5-methyl-5-hexen-2-ol was detected (Rt, 7.930 minutes). 2- methylhexadecanal, an aldehyde was detected (Rt:13.967 minutes:1.6%) in this work while hexadecane (Rt: 8.379 minutes), an alkane among others were detected by Amin et al. [33]

4.0. Conclusion

The research findings have indicated that methanol extract of sweet orange possess significant antifungal activity against *E. floccosum* and *C. albican* and the extracts inclusion in boby creams and lotions may be considered for further studies

Conflict of Interest

The authors declare no conflict of interest in this work.

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