JMPHTR 8 (2021) 1-7 ISSN 2053-1826

Journal of Medicinal Plant and Herbal Therapy Research
www.bluepenjournals.org/jmphtr

Effects of 5-hydroxymethylfurfural isolated from *Cola hispida* on oral adenosquamous carcinoma and MDR *Staphylococcus aureus*

doi.org/10.33500/jmphtr.2021.08.001

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Article History
Received 29 April, 2021
Received in revised form 30 May, 2021
Accepted 03 June, 2021

Keywords:
Cytotoxicity, Oral adenosquamous carcinoma, *Staphylococcus aureus*, 5-hydroxymethylfurfural.

Article Type:
Full Length Research Article

ABSTRACT

Phytochemicals and derivatives present in medicinal plants are promising alternatives to improve treatment efficacy in human diseases. Most drugs in clinical use today owe their origin to plant based complementary medicine. No effective drugs in the treatment of oral cancer and multidrug-resistant (MDR) pathogens. This study was therefore aimed at isolating and identifying molecules with cytotoxic activity on cancer cell line and antimicrobial inhibitory activity from medicinal plant identified from Nigerian ethnomedicine. Ethyl acetate fraction of *Cola hispida* Brenan and Keay seed (FHI 111321) was subjected to chromatographic separations to isolate and purify bioactive compounds. Structures of isolated compounds were determined by spectroscopic analyses. Cytotoxicity assay was performed on isolated compound against oral adenosquamous carcinoma cell line (CAL-27) in a 96 well plate using MTT assay at 250 µg/mL. 5FU was used as standard. Inhibition of fully grown MDR *Staphylococcus aureus* strains (NCTC 13277) in diluted Mueller Hinton Broth (MHB) media was done using Micro-Plate AlamarBlue Assay (MABA) at 500 µg/mL. Gentamycin was used as a standard. 5-hydroxymethylfurfural (1) was isolated from *C. hispida* seed for the first time. Anticancer activity of 5-hydroxymethylfurfural on oral adenosquamous carcinoma cell line (CAL-27) had maximal inhibitory concentration (IC_{50}) as 68.81±4.00 µg/mL when compared to standard 5FU with IC_{50} of 2.41 µM. The minimum inhibitory concentration (MIC) of 5-hydroxymethylfurfural against MDR *S. aureus* (nctc 13277) was found to be 250 µg/mL at 71.96% inhibition when compared to standard gentamycin at 5 µg/mL (95.00% inhibition). 5-hydroxymethylfurfural could serve as a potential lead for novel drug development for the management of oral cancer and inhibition of multi drug resistance strain of *S. aureus*.

INTRODUCTION

Cola hispida Brenan and Keay is a shrub or tree up to 40 ft. high, can be found in a forest. Their leaves is up to 30 cm long and 24 cm broad. Flowers greenish-yellow inside with brown pubescence outside, inside varying to reddish-pink. It has a fruit that looks like a billy goat testicles clustered together. It is orange in colour when riped containg seeds in white coat typical of the Cola genus. The root grind to powder mixed with palm oil is rubbed unto the

skin for treatment of skin infections (Burkill, 2000). Medicinal plants have been used for the management or treatment of various disease ailments from time immemorial (Sevindik et al., 2017; Mohammed et al., 2019) Phytochemicals and derivatives present in plants are promising options to improve treatment efficiency in human diseases (Mohammed et al., 2020). Most drugs in clinical use today originated from natural products, where they served as drug precursors, templates for synthetic modification and pharmacological probes (Newman and Cragg, 2016; Mohammed et al., 2021). The vinca alkaloids (Vinblastine, Vincristine, Vindesine, Vinorelbine) are examples of phytochemicals that has been reported to have anticancer effects on non-small-cell lung carcinoma, breast, lung, leukemia, Hodgkin and non-Hodgkin lymphomas, testicular carcinoma, Kaposi's sarcoma, and second-line transitional cell carcinoma of the urothelium by Inhibiting microtubule polymerization and assembly, leading to metaphase arrest and cell death (Martino et al., 2018).

On the other hand, there are examples of medicinal plants and molecules of natural origin that has exhibited various degrees of antimicrobial activities. Such includes *Mentha longifolia* (L.) Hudson subsp. *longifolia* and *Scorzonera papposa* ethanol extracts reported to exhibited antimicrobial activity on *Staphylococcus aureus* ATCC 29213 (Sevindik et al., 2017; Mohammed et al., 2020), tomatidine an alkaloid has been proven to be effective against *S. aureus* when grown in presence of *P. aeruginosa* (Mitchell et al., 2011). Sanguinarine, a benzophenanthri-dine alkaloid derived from the rhizomes of *Sanguinaria canadensis* L., has broad antimicrobial activity by perturbing bacterial FtsZ Z-ring formation and inhibiting bacterial cytokinesis (Beuria et al., 2005; Kelley et al., 2012). Isothiocyanates are organosulfur compounds found in the Brassicaceae family have shown to be active against gram-negative and gram-positive bacteria such as *S. aureus* (Dias et al., 2012; Park et al., 2013).

Cancer is one of the major threats to public health both in the developed world and progressively in the developing world. Cancer is the second most common cause of death in developed countries. Cancer accounts for 7.1 million deaths in year 2003 and it is projected the overall number of new cases will rise by 50% in the next 20 years (WHO, 2003; Sevindik, 2020). Adenosquamous carcinoma is an unusual variant of squamous cell carcinoma of the oral cavity. It was first described by Gerughty as a type of malignant salivary gland tumor (Gerughty et al., 1968). The World Health Organisation classification of head-and-neck tumors in 2017 defined Adenosquamous carcinoma as "a malignant tumor that arises from the surface epithelium and histologically shows both squamous and glandular differentiation (El-Naggar et al., 2017). It is a high-grade, aggressive, highly infiltrating and an extremely rare variant of malignant epithelial neoplasm with a high metastatic rate (80%) exhibiting poor prognosis (Thanakappan et al., 2015; Sravya et al., 2016). The occurrence of oral cancer is particularly high among men, the eighth most common cancer worldwide. Incidence rates for oral cancer vary in men from 1 to 10 cases per 100 000 population in many countries. In south-central Asia, cancer of the oral cavity ranks among the three most common types of cancer. In India, the age standardized incidence rate of oral cancer is 12.6 per 100.000 population. It is remarkable that sharp upsurges in the incidence rates of oral/pharyngeal cancers have been reported for several countries and regions such as Germany, Scotland, Denmark, France, central and Eastern Europe and to a lesser degree Australia, Japan, New Zealand and the USA (Stewart and Kleihues, 2003; Petersen, 2003). The use of tobacco and consumption of excessive alcohol have been projected to account for about 90% of cancers in the oral cavity; the oral cancer risk surges when tobacco is used in combination with alcohol or areca nut (Reibel, 2003).

Overuse and improper consumption and application of antibiotics have driven the prompt emergence of multidrug-resistant (MDR) pathogens (Carlet et al., 2011). Antimicrobial resistance increases the disease, death, length of hospitalization and healthcare costs. Gram-positive bacteria, *S. aureus* have become a major global healthcare problem because of the fast rate at which it develops resistance to common antimicrobial agents. Pendleton et al. (2013) reported some emerging multi drug resistance bacteria or pathogens increasingly escape the effects of antibiotics (*Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.).

It is indisputable that antibiotic resistance is life intimidating and patients with infections caused by resistant pathogens have higher death rates and increased human misery. *S. aureus* has developed resistance to several antimicrobial drugs, including second- and third-line drugs. Only a few drugs, such as vancomycin (a glycopeptide), daptomycin (a lipopeptide), and linezolid (an oxazolidinone) have been approved for the treatment of serious infections caused by Multidrug-Resistant *S. aureus* (Brandon and Dowzicky, 2013). Available treatment options for serious invasive disease owed to *S. aureus* are limited because of amassed antimicrobial resistance.

New drugs that can inhibit proliferated cancer cells and antibiotics capable to defeat MDR pathogens especially from medicinal plants are desperately needed hence this research. This study was therefore, aimed at isolating and identifying molecules with cytotoxic activity on cancer cell line and antimicrobial inhibitory activity from medicinal plant identified from Nigerian ethnomedicine.

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MATERIALS AND METHODS

Chemicals and reagents

MTT dye, Formazan crystals, DMSO, 5FU (Fluororacil), Mueller Hinton Broth (MHB), Alamarblue dye (Alfa Aesar) and other chemicals were of analytical grade.

Collection, authentication and extraction of plant material

Plant materials

The plant *C. hispida* (Malvaceae) seed was obtained from Okura-Ofante, Dekina Local Government Area of Kogi State in the month of March, 2017. Plant was authenticated at Forestry Herbarium Ibadan (FHI) by Chukwuma Emmanuel where voucher specimen was deposited as FHI 111321.

Preparation of plant extract

The plants were air dried for three weeks and pulverized. 11 Kg of powdered sample of *C. hispida* seed were macerated with 100% methanol for 72 hours. The filterate was concentrated *in vacuo*. The methanolic crude extract was suspended in water and partitioned successively with n-hexane, dichloromethane, ethyl acetate and aqueous methanol using a separating funnel.

Isolation of bioactive molecules

The ethyl acetate fraction (70 g) of *C. hispida* was chromatographed using column chromatography on 600 g column silica gel (60-200 mesh size). The gradient elution started with n-hexane (100%, 2000 mL), hexane: dichloromethane (9:1-1:9, 2000 mL each) to yield 116 sub fractions. The solvent system hexane: dichloromethane (1:9) eluted compound 1 as yellow eluate (oily in nature).

MTT Assay (Antiproliferative assay)

Cytotoxicity assay was performed by seeding 10,000 cells (CAL-27) per well in a 96 well plate. Followed by an incubation period of 24 h the cells were treated with compound at different concentrations (250-1.95 µg/mL). After incubation of 48 h at 37°C in CO_2 incubator, 0.5 mg/mL MTT dye was added in each and was further incubated for 4 h. Later Formazan crystals were solublized in 100 µL of DMSO. The absorbance was recorded at 570 nm using Spectrophotometer. 5FU was used as standard. The half IC_{50} was calculated using the software, EZ-Fit Enzyme Kinetics by Perrella Scientific (Farooq et al., 2017).

Micro-plate AlamarBlue assay (MABA)

Stock solution 20 mg/mL of compound was prepared in 100% DMSO and serially double fold diluted in Mueller Hinton Broth in 96 well plate ranging concentration from (500, 250, 125, 62.5, 31.25 µg/mL) such that the final volume becomes 100 µL (compound and media). Fully grown MDR *S. aureus* strains (NCTC 13277) was 1000 times diluted in MHB media and 100 µL of this diluted bacterial media were added to all previous wells such that final volume (200 µL) would contain bacterial load approximately (0.5-1.0 × 10^6 CFU/mL). Positive control contains only media and bacteria. Each concentration was run in triplicates. Plates were sealed and incubated at 37°C for 18 - 22 h. Next day, all wells were visually checked to confirm the growth of bacteria. Soon after noticing the clear and turbid wells, 20 µL of 0.02% Alamarblue dye (Alfa Aesar) was added in each well, and incubated in a shaking incubator at 80 rpm, and 37°C for 2 h. Change in color of Alamar dye from blue to pink indicated the growth of bacteria strains. For quantitative analysis, absorbance was recorded at 570 nm and 600 nm in Multiskan™ GO spectrophotometer (ThermoScientific, USA).

Percentage difference in reduction of Alamar Blue between treated and control cells were calculated using the formula mentioned by Lancaster et al. (1996).

Statistical analysis

All data will be expressed as mean ± S.D. and of triplicate parallel measurements. The half IC_{50} was calculated using the software, EZ-Fit Enzyme Kinetics by Perrella Scientific.

RESULTS AND DISCUSSION

Structural elucidation of isolated compound

The compound has the molecular formula: $C_6H_6O_3$, melting point (M.p): 112.5°C-113.2°C, Mass (m/z): 126.1 g/mol [M+]. Its low resolution electron impact (EI) mass spectrum on JEOL 600H-1 instrument showed major fragmentation at m/z (%): 126.1 (63.9), 109.0 (19.2), 97.0 (100.0), 81.0 (5.8), 69.1 (45.2), 53.0 (22.7), 41.0 (97.0). UV (MeOH) λ/nm (log ε): 229.00 nm (A 1.659), 262.00 nm (A 2.527), 271.00 nm (A 2.553), IR (γ_{max} ^{NEAT} cm-1): 3389.7, 2930.5, 2846.2, 1678.5, 1522.9, 1398.9, 1281.4, 1192.8, 1023.6, 812.6, 776.8, 616.7. Structure has been assigned to the substance 5-hydroxymethylfurfural (Figure 1) based on the physical data and spectroscopic analysis (UV, IR,

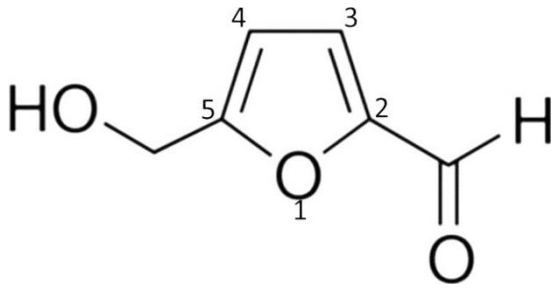

Figure 1. Compound 1 (5-Hydroxymethylfurfural).

Table 1. ^{1}H-NMR (AVANCE-III-AV-400 MHz) and ^{13}C-NMR (AVANCE NEO 300 MHz) chemical shift values for 5-Hydroxymethylfurfural (ECH-9I) recorded in CD3OD.

^{1}H-NMR	^{13}C-NMR	HMBC
9.51 (s, 1H, CHO, H-2)	179.43	57.57, 124.89, 163.11
7.37 (d, 1H, J = 3.6 Hz, H-3)	124.89	110.88, 153.76, 163.11, 179.43
6.57 (d, 1H, J = 3.6 Hz, H-4)	110.88	57.57, 124.89, 153.76, 163.11, 179.43
4.60 (s, 3H, CH_2OH, H-5)	57.57	110.88, 124.89, 153.76, 163.11

Chemical shift values are in δ (ppm); Coupling constants are in Hz.

Table 2. TLC profile of 5-Hydroxymethylfurfural isolated from *C. hispida*.

Compound	Retarding factor (R_f) (cm)	Solvent system	Weight (mg)
5-Hydroxymethylfurfural	0.65	Hexane:Ethyl acetate (3:7)	125

EI-MS, ^{1}H-NMR and ^{13}C-NMR) and by comparison with reported literature (Prasenjit and Paresh, 2013) (Tables 1 and 2). 5-Hydroxymethylfurfural is a member of the class of furans that is furan which is substituted at positions 2 and 5 by formyl and hydroxymethyl substituents, respectively isolated from *C. hispida*. ^{1}H- NMR reveals the presence of an aldehyde (CHO) which resonate around 9.51 ppm as a singlet, the methylene hydroxygroup (OH) resonate around 4.60 ppm as a singlet, while the furan ring resonate as a doublet, 1H each with a coupling constant of 3.6 Hz around 7.37 ppm and 6.57 ppm, respectively. ^{13}C- NMR-(δ, AVANCE NEO 300 MHz CD3OD): showed the presence of six carbons. Polarization transfer experiments (DEPT) were carried out with last polarization angles at 135°, 90° to determine the multiplicity of each carbon atom. DEPT 135° reveals the presence of one methylene (CH_2) at 57.57 ppm and five methine groups (CH) which resonate around δ 179.4, 163.1, 153.6, 124.9 and 110.8. DEPT-HSQC reveals that proton 9.51 ppm is attached to carbon at 179.43 ppm (CHO), 7.37 ppm doublet with coupling constant of 3.6 Hz is attached to carbon at 124.8 ppm, 6.57 ppm also a doublet with coupling constant of 3.6 Hz is attached to carbon at 110.8 ppm which account for the furan ring and proton 4.60 ppm attached to carbon 57.57 ppm (OH). The molecule consists of a furan ring, containing both aldehyde and alcohol functional groups. This compound is reported for the first time in the species *C. hispida*.

Evaluation of anticancer activity (MTT assay)

Adenosquamous carcinoma is a rare, aggressive malignant epithelial neoplasm of the oral cavity. Siar and Ng (1987) reported that, in the head and neck region, larynx is the most common site of occurrence for adenosquamous carcinoma with 48.4% followed by the oral cavity at 30%. The floor of the mouth, tongue, alveolus, palate and upper lip are the most common locations within the oral cavity where the oral cancer can happen. The disease is categorized by continual proliferation of cells in the human body with the failure to be stopped or controlled. Therefore, forming tumours of malignant cells with the potential to be metastatic

(Ochwang'i et al., 2014). Adjuvant chemotherapy, radiotherapy and chemically derived drugs are the current treatments options for head and neck cancers. Treatments such as chemotherapy can put patients under a lot of strain and further damage their health. Therefore, there is a focus on using alternative treatments and therapies against cancer (Cancer Research UK, 2014).

Currently, alternative medicine has promising sources of new anticancer molecules which is obvious from the fact that the bulk of existing anticancer drugs are either natural products or their phytochemicals (Newman and Cragg, 2007; Cragg et al., 2009; Li and Vederas, 2009). Interestingly, there is an increased interest in last few decades on the use of medicinal herbs for the treatment or management of human diseases such as cancer because of their fewer side effects compared to conventional chemotherapy (Dev, 2010). There are natural compounds that have been reported to have potential to treat oral cancer. Thymoquinone isolated from seeds of *Nigella sativa* L. (Ranunculaceae) has been reported to increase the expression and activation of p53 within T28 oral cancer cells (Abdelfadil et al., 2013), Nimbolide is a natural bioactive compound isolated from neem tree has been reported to induce apoptosis and inhibit cell proliferation in an animal model for oral carcinogenesis (Harish et al., 2010), Aloe emodin a natural compound has been reported to regulate growth of oral cancer cells by disrupting DNA repair mechanism (Chen et al., 2010). GC-MS analyses, revealed 5-hydroxymethylfurfural (23.1%) as major components in the *Pyrrosia piloselloides* (L.) M.G. Price methanol extract which exert potent anti-proliferative effect on hela human cervical carcinoma cell line (Sul'ain et al., 2019), *Lactifluus rugatus* and *Allium calocephalum* methanol extract has been reported to have antiproliferative activity on lung cancer cell line A549 (Sevindik, 2020; Mohammed et al., 2019). GC-MS analyses showed that the highest percentage of compound identified in the extract of *Garcinia dulcis* (Roxb.) Kurz flesh was hydroxymethylfurfural and 3-methyl-2,5-furandione, together with xanthones and flavonoids which could synergistically contribute to the observed effects in inducing cytotoxicity and apoptosis in HepG2 liver cancer cell line (Abu et al., 2015).

Russel et al. (2019) reported the use of 5-hydroxymethylfurfural or 1,10-phenanthroline as a linker for cancer cell-specific drug delivery system that contains β-cyclodextrin as a drug carrier and folic acid as a targeting ligand because folic acid receptors are highly expressed in a variety of tumor types.

Tuyen et al. (2016) reported the cytotoxicity of 5-hydroxymethylfurfural alongside stipudiol and panaxytriol isolated from the rhizomes of *Panax stipuleanatus* H.T.Tsai and K.M.Feng against KB cell line. Zhao et al. (2013) revealed the antiproliferative activities of 5-hydroxymethylfurfural on human melanoma A375 cells, L02 cells and SW480 cells. 5-hydroxymethylfurfural has been reported to form part of several anticancer compositions with synergistic effect and can selectively inhibit the cell cycle of cancer cells while showing no toxicity to normal cells, thereby inducing apoptosis (Seo et al., 2014; Li and Xu, 2013; Suh et al., 2012).

In an attempt to find a safer, affordable and effective treatment option, we investigated here the use of 5-hydroxymethylfurfural against oral adenosquamous carcinoma cell line, one of the active components of *C. hispida* plant, traditionally used for various medicinal purposes. The molecule 5-Hydroxymethylfurfural showed potential antiproliferative and cytotoxicity against on oral adenosquamous carcinoma cell line (CAL-27) with IC_{50} of 68.81±4.00 µg/mL as compared to standard 5FU (Fluororacil) with IC_{50} of 2.41µM (Table 3). The results of the present study indicate that 5-hydroxymethylfurfural have potential anti-proliferative property on CAL-27 cells and can be used as pharmaceutical case study for oral cancer treatments.

Evaluation of antibacterial activity

The discovery of antibiotics has been one of the most significant advances in the modern medicine. The biological pressure imposed by the continuous exposure to different antibiotics during clinical application has led to the cumulative acquisition of resistant traits in major human pathogens resulting in MDR bacteria, which are practically impossible to treat. The Gram-positive bacterium *S. aureus*, a member of the bacterial class "Bacilli", is historically the most notorious superbug (Peacock and Paterson, 2015).

S. aureus is an extremely successful human pathogen, which is largely due to its versatility and mammoth capacity to acquire antibiotic resistances. In the pre-antibiotic era, death associated with *S. aureus* exceeded 80% (Skinner and Keefer, 1941). Today, it is a frequently isolated pathogen causing serious, invasive infections such as soft tissue infections, endocarditis, osteomyelitis, bacteremia, septic arthritis, and nosocomial pneumonia (Sader et al., 2005).

From the study conducted, the minimum inhibitory concentration (MIC) of 5-hydroxymethylfurfural against MDR *S. aureus* (NCTC 13277) was found to be 250 µg/mL at 71.96% inhibition when compared to standard gentamycin with MIC at 5 µg/mL (95.00% inhibition) (Table 4). This finding is in agreement with Vijayakumar et al. (2018) who reported the Antiquorum sensing and biofilm potential of 5- hydroxymethylfurfural against gram-positive pathogens such as *Chromobacterium violaceum*, *Streptococcus pyogenes*, *S. mutans*, *S. aureus* and *S. epidermidis*. 5-hydroxymethylfurfural has demonstrated to be efficient against infections caused by multi-drug resistance strain of bacteria (*S. aureus*).

Table 3. Anticancer activity of 5-Hydroxymethylfurfural on oral adenosquamous carcinoma cell line (CAL-27).

Compounds	CAL-27 (IC_{50})
5-hydroxymethyl furfural	68.81±4.00 (µg ± S.D)
5FU (STD drug)	2.41µM

Table 4. Minimum inhibitory concentration (MIC) of 5-hydroxymethylfurfural against MDR *S. aureus* (NCTC 13277).

Compounds	*S. aureus* (NCTC 13277)	
	MIC	% inhibition
5-hydroxymethylfurfural	250 µg/mL	71.96%
Gentamycin (STD drug)	5 ug/mL	95.00%

Conclusion

5-hydroxymethylfurfural was isolated from *C. hispida* for the first time and could serve as potential leads for novel drug development for the management or treatment of oral cancer and inhibition of multi drug resistance strain of *S. aureus*.

ACKNOWLEDGEMENTS

Authors would like to acknowledge Third World Academy of Science (TWAS) and International Centre for Chemical and Biological Sciences (ICCBS), University of Karachi for funding part of this research.

Abbreviations

MIC, Minimum Inhibitory Concentration; **MDR,** multidrug-resistant; **MABA,** micro-plate AlamarBlue Assay; **MHB,** Mueller Hinton Broth; **5FU,** Fluororacil; **DMSO,** Dimethyl sulfoxide; **CAL-27,** oral Adenosquamous carcinoma cell line; **NMR,** nuclear magnetic resonance; **HSQC,** heteronuclear single quantum coherence; **IR,** infrared spectroscopy; **FT-IR,** fourier-transform infrared spectroscopy; **UV,** ultraviolet-visible spectroscopy; **HMBC,** heteronuclear multiple bond correlation; **EI-MS,** electron impact mass spectrum; **DEPT,** distortionless enhancement by polarization transfer; **TLC,** thin layer chromatography; **MP,** melting point.

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