AWARD NUMBER: W81XWH-19-1-0785

TITLE: Anti-cancer Efficacy of CBD Pure Isolates and Commercially Available Water-Soluble CBD in Colorectal Cancer

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REPORT DATE: October 2020

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE		*Form Approved OMB No. 0704-0188*

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
Oct 2020	Annual	15 Sep 2019 – 14 Sep 2020

4. TITLE AND SUBTITLE

Anti-cancer Efficacy of CBD Pure Isolates and Commercially Available Water-Soluble CBD in Colorectal Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH19-1-0785

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S) Dr. Sarah Daron-Mathis

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

According to the most recent information from the American Cancer Society (ACS), colorectal cancer (CRC) is the 3rd most common cancer in the United State. Moreover, rates are rising in younger age groups. Amongst Military Veterans, approximately 13,000 cases of CRC have been reported from 2009-2012. Critically, while early-stage cancers respond well to treatment, metastatic colorectal cancer has a 5-year survival of only 14%, emphasizing a need for new therapeutic approaches. CBD, which is a non-psychotropic cannabinoid from the Cannabis sativa plant, has been studied since the 1970s. There is some evidence that it has anti-tumorigenic properties such as slowing of tumor progression, apoptosis induction, and proliferation inhibition although the studies are few. However, CBD like many other drugs, has low water solubility leading to poor bioavailability. Several companies have apparently developed what is being called Water-Soluble CBD, which could theoretically improve bioavailability. This water-soluble CBD would allow for higher concentrations of CBD to be available to a tumor and a dosage regimen that maintained optimal levels for anti-cancer activity to be identified. Given the availability of such agents, the claims being made for their efficacy, as well as the dire need for effective treatments for CRC, this study is designed to determine the efficacy, safety, and validity of water-soluble CBD compounds in colon cancer in vitro and in vivo studies.

15. SUBJECT TERMS None Listed					
16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF**	**18. NUMBER OF**	**19a. NAME OF RESPONSIBLE PERSON** USAMRMC
a. REPORT Unclassified	**b. ABSTRACT** Unclassified	**c. THIS PAGE** Unclassified	Unclassified	12	**19b. TELEPHONE NUMBER** *(include area code)* 304-730-5456

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Introduction

Cannabidiol (CBD) has become very popular since the Farm Bill Act of 2018 and while it has been studied since the 1970s, the research has been quite spare. There has been is evidence of CBD being anti-tumorigenic by slowing progression, inducing apoptosis, and having anti-proliferative effects in various cancer cells. However, CBD like many other drugs, has low water solubility that leads to poor bioavailability, with bioavailability based on route of administration reported as: 5% oral, 13% sublingual, 30% inhaled. Several companies have apparently developed what is being called Water-Soluble CBD, and state without much evidence that it has improved bioavailability taken orally. This water-soluble CBD, if proven true, would allow for higher concentrations of CBD to be available to a tumor, and a dosage regimen that maintained optimal levels for anti-cancer activity to be identified. Given the availability of such agents, the claims being made for their efficacy, as well as the dire need for effective treatments for Colorectal Cancer (CRC), this study is designed to determine the efficacy, safety, and validity of water-soluble CBD compounds in colon cancer *in vitro* and *in vivo* studies.

Keywords

Anti-proliferative

Anti-tumorigenic

Bioavailability

Cannabidiol (CBD)

Colorectal Cancer (CRC)

Pharmacokinetic (PK)

Accomplishments

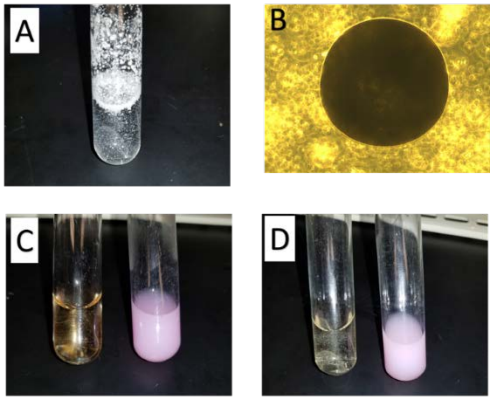
- **What were the major goals of the project?**
    - **Major Task 1-** Obtain regulatory approval for animal work
        - IACUC approval July 21, 2020
        - ACURO approval – Submitted and Awaiting
    - **Major Task 2-** Determine the anti-tumor effects of CBD and water-soluble CBD on colon cancer cells
        - Analyze all CBD compounds using Mass spec. February 28, 2020
        - Evaluate CBD effects on proliferation, cell death, clonogenic survival, migration and invasion in 5 human and 2 mouse colon cancer cell lines.
    - **Major Task 3-** Measure the *in vivo* bioavailability and pharmacokinetic (PK) parameters of the water-soluble CBD and CBD isolates by oral administration.
        - **Not started**
    - **Major Task 4-** Assess the anti-tumor effects in vivo, using both a colitis-associated cancer model (AOM/DSS) and a de novo tumorigenesis model (*Apc*$^{min/+}$)
        - **Not Started**
    - **Major Task 5**- Analyze data and prepare manuscript
        - **Not Started**

- **What was accomplished under these goals?**
    - **Major Task 1**
        - Obtain regulatory approval for animal work IACUC approval July 21, 2020. ACURO approval was submitted on August 21, 2020 and is awaiting approval so that mice can be purchased.
    - **Major Task 2**
        - Determine the anti-tumor effects of CBD and water-soluble CBD on colon cancer cells
        - Subtask 1- Analyze all CBD compounds using MS

Isolate is crystalized pure CBD known in the industry as Isolate. MI, TN, and NC are three water-soluble CBD formulations from three different companies and using the only identifier as the state from which they are from.

	Provided COA	Results of LCMS
Isolate	100%	84.76%
MI	11.50%	11.53%
TN	41.82%	8.77%
NC	10.62%	6.99%

Isolate was found lower than the third-party lab test but this happens to most isolated CBD because it absorbs water in the air and if not, completely sealed moisture contaminates the purity. Still comparable to what you would find in a dispensary. MI was very much on target with what their third-party sent.



When I spoke with the company in TN they said that they think there was a mix up and gave me a new sample. NC sample was a little low went I ran it but again over time the moisture could contaminate.

This also led to determining the best way to solubilize CBD Isolate. I found that in pure DMSO at 1M or even 10mM the drug would form spheres in culture. I tested mixing with EtOH but this caused necrosis and finally determined that 1M Isolate in DMSO 4 parts to 6 parts media to 10mM concentration would work and stay in solution. While PBS worked well for the water-soluble CBD formulations.

Figure 1, Solubility of different CBD formulations.
A. CBD Isolate at 1M concentration in PBS.
B. CBD Isolate in DMSO mixed with Cell Culture Media at 100uM under microscope at 10X.
C. CBD Isolate in EtOH at 1M (left) CBD Isolate at 10mM in 4:6 ratio of EtOH and Media.
D. CBD Isolate in DMSO at 1M (left) CBD Isolate at 10mM in 4:6 ratio of DMSO and Media.
E. 1. CBD water-soluble solution from NC at 1mM. 2. CBD water-soluble solution from MI at 1mM. CBD water-soluble solution from TN at 1mM.

- Subtask 2 – Evaluate CBD effects on proliferation, cell death, clonogenic survival, migration and invasion. On 5 human colon cancer lines and 2 mouse lines.
  - DLD-1 –Conclusions Below

DLD-1 cell line was first to be tested due to its efficiency in growth and because of data shown in Jeong et at 2019 experiments. They reported using DLD-1 cells in a WST-8 assay, Clonogenic assay, and Annexin V assay all showing an IC50 of 6uM CBD Isolate. I followed the concentrations, cell numbers and length of time used in their experiments to replicate this data. Using 8000 cells per well in 96 well, 24 hr treatment, followed by MTS assay or CyQuant assay. However, after many different attempts with solvents, cells numbers, time of treatments, and different concentrations this was reevaluated and found that the IC50 for Isolate was about 60uM. MTS assay and the more sensitive CyQuant assay, that shows a 40uM IC50 both confirmed this data with replicates of 3 being done.

Migration assays were then performed. This assay looks into both growth of cells treated and the cells ability to migrate toward each other to close a gap. Cells were placed in 6 well plates and were seeded at 100% confluency. Once cells were attached, the plate was scratched down the center with a 1ml pipette tip. And then treated with different formulations of the CBD (Isolate, MI, NC, and TN) or controls (media or media with DMSO), pictures were taken after 24 hrs. The migration assays showed that at 10uM Isolate CBD did not migrate significantly with p value > 0.05. This is the same for the water-soluble compounds MI and NC. However, TN cells showed growth and migration at 10uM with a p value <0.05. When cells were treated with 30uM both the Isolate and MI had reduced migration and growth compares to their respective controls p value <0.05 and NC showed no change, and TN still showed growth and migrations with a p value <0.05. Cells were also treated at

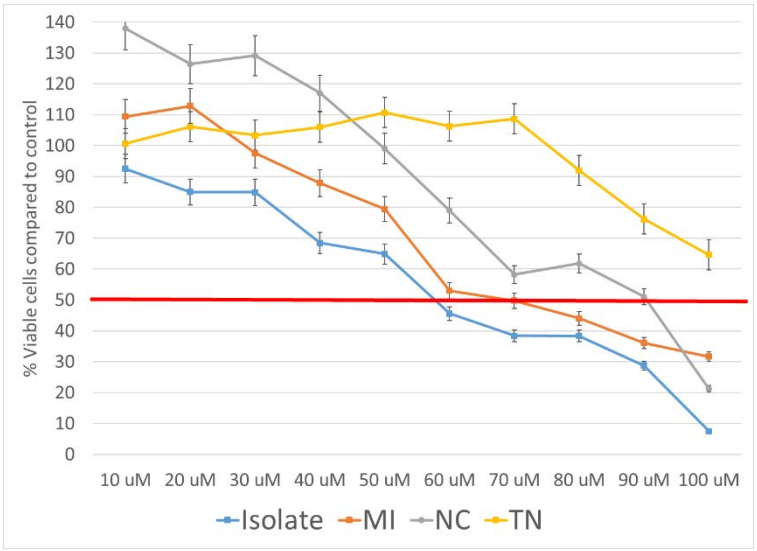


Figure 2. MTS assay of different CBD formulations DLD-1 cells after 24 hr. MI, NC, and TN are three different water-soluble formulations from companies within those states. Graph shows treatments depicting the percent viable cells compared to the control, red line indicates the IC50. Isolated IC50 found to be at 60uM P<0.005. MI IC50 found to be at 70uM P<0.05. NC IC50 found to be at 90 M P<0.005. TN never reached IC50. All treatments replicated 3 times, N=3.

60uM and 90uM and Growth and migration was increasingly inhibited with Isolate, MI and NC but had little to no effect on TN. All these assays were measured 3 times with 4 replications. This data confirmed the above MTS assay showing that the water-soluble CBD from TN had no effect.

The clonogenic assay was performed by seeded cells at 50% confluency in 6 well plates and the following day treating cells with 30, 60, or 90uM of Isolate, MI, NC, TN, DMSO, or nothing in media for 24 hours. Cells were collected after the 24 hrs being careful not to disregard any dead cells and using trypan blue did a live/dead cell count. DLD-1 cells were seeded at 250 cells per well and given untreated media for 2 weeks then stained with crystal violet and colonies were counted. This data confirmed again that at 30uM, DLD-1 cells showed little response to treatments of 30uM, but by 90uM no colonies survived Isolate, MI, and NC whereas, TN still showed very little response n=3.

EDU assay show the proliferation of cells by labeling the DNA at the start of the experiment and with each doubling of cells the fluoresces intensity degreases. Cells were seeded 8000 cells per will in 96 well and of different CBD formulations were added after cells attached along with EDU for 24 hrs. The Isolate found reduction of proliferation significantly (p value<0.005) at 30uM = 45% reduction, 60uM = 60% reduction, and 90uM = 60% reduction of proliferation compared to the control, as well as MI 90uM = 62% reduction, and NC 90uM = 52% reduction, and a 27% reduction in TN 90uM (p value<0.05) n=3.

In Progress is the Annexin V Assay which has been run on a Guava flow cytometer to determine where cells are in early apoptosis, late apoptosis or necrosis. This analysis is underway. Also Cleaved Caspase 3 Western Blots, the protein collected but Western Blots not run yet. These will be run once all protein samples from all lines are collected.

- HCT-116- Conclusion below

HCT-116 cell line was the second human cell line to be tested due again this was because of the data shown in Jeong et at 2019 experiments. They reported using HCT-116 cells in a WST-8 assay, Clonogenic assay, and Annexin V assay all showing an IC50 of 4-6uM CBD Isolate. Again, using the same number of cells and length of treatment but due to the information gathered from DLD-1 higher concentrations were used to determine the IC50 of HCT-116 which was found to be at 70uM (p value <0.05 n=3). This assay also evaluated the water-soluble formulations and found MI IC50 was at 50uM (p value <0.05 n=3). However, the response for MI seems to plateau at 60uM.

The migration assay of different CBD formulations HCT116 cells after 24 hr. MI, NC, and TN done following same protocol as DLD-1 for 10, 30,60, and 90uM. This showed that both Isolate and MI had a significate reduction of migration in 10uM (p value <0.05 n=3). The 30uM, 60uM, and 90uM also showed a significant increase on Isolate, MI, NC and even TN (except 90uM). This led to reevaluations of MTS assay and replicates are in progress as well as the CyQuant assay for confirmation.

EDU assay show the proliferation of HCT-116 cells that were seeded 10000 cells per will in 96 well and of different CBD formulations were added after cells attached along with EDU for 24 hrs. The Isolate found reduction of proliferation significantly however to a lesser extent then DLD-1, (p value<0.05) at 30uM = 15% reduction, 60uM = 20% reduction, and 90uM = 35% reduction of proliferation compared to the control, as well as MI 90uM = 28% reduction, and NC 90uM = 35% reduction, and a 20% reduction in TN 90uM (p value<0.05) n=3.

In Progress is the Annexin V Assay which has been run on a Guava flow cytometer and analysis is underway. Also Cleaved Caspase 3 Western Blots, the protein collected but Western Blots not run yet. These will be run once all protein samples from all lines are collected. The Clonogenic Assay was started in the first week of October and should have results by end of the month.

- HT29 – Conclusions of progress below

HT29 cell line was the third human cells line to be tested and in Jeong et at 2019 experiments they only looked at a WST-8 assay. Showing that the IC50 was only slightly lower then 6uM of the DLD-1 cells line. However, they did not give much detail to how many cells were used per well and no other experiments were done in their study. I have begun to look at the MTS assay looking at 10uM to 100uM and found

that the IC50 is over this amount. I have been reevaluating this assay and will be continuing to work on this throughout this month and next. Once the IC50 has been determined the Rest of the experimental assays will follow.

- Caco-2 – Have not started, having a difficult time recovering cyro-stocks
- SW620 – Planned to do this last because it requires growth without CO2
- CT26 – In progress

CT26 are a mice colon cancer cell line and were first experimented with in February along with DLD-1. This was because both DLD-1 and CT26 use the same media and seemed reasonable to do these experiments alongside of each other. When doing the CT26 cells MTS assay the proper solvent method had not been developed yet. Also, when working on the migration assay I noticed that the cells didn't so much as migrate into the area of the scratch but they seem to detach during proliferation and reattached in areas that had available space. This needs to be reconsidered how to best use and analysis this assay. This is being reevaluated in the upcoming month after a conversation how to best address this with my committee.

- MC28- Have not started. Still need cell line.

Based on initial cell experiments I need to reprioritize my efforts before proceeding with PK/animal experiments to instead understanding the mechanism of action of CBD on these cells and why this may be different then the literature suggests. Also, the fact that cell lines appear to be responding differently may indicate that there are potential sub-classes of CRC that are more sensitive to CBD, and should be explored further before proceeding with any mice studies. This refocusing will allow for more precision in the amount of CBD given to the mice and by nailing down the mechanism by which these cells are responding it will also have the potential to influence any downstream animal studies and/or identification of new biomarkers that can be used to track response of tumors in mice.

- **What opportunities for training and professional development has the project provided?**
  - Development of mentorship for an undergraduate student for undergraduate research.
  - Internal grant proposal submitted for MTSU Special Project Grant.
  - Guest lecturing "Nutraceuticals & Hemp", "CRISPR in Agricultural Biotechnology" and "Principles and Application of Flow Cytometry" at the School of Agriculture at MTSU.
  - Mentorship Committee to guide my grant work and professional development.

The College of Basic and Applied Sciences at MTSU has provided extra resources to support my grant work. My mentorship committee consists of three Biology faculty and one Agriculture faculty. The Committee is available for questions and advice on my research and will schedule regular monthly meetings with me to review my research progress and discuss any research questions.

- **How were the results disseminated to communities of interest?**
  - Poster presentation "Anti-cancer efficacy of CBD Pure Isolates and Commercially Available Water-Soluble CBD in Colorectal Cancer", MTSU Scholars Week, April 2020.
  - Oral presentation "Hemp and Health", Pick Tennessee Conference, February 21, 2020.
    - Hosted by Tennessee Department of Agriculture and Tennessee Organic Growers Association (TOGA).
  - Many scheduled conferences were canceled due to Covid-19 in year one, but further virtual conferences and opportunities will be sought during year two.

- **What do you plan to do during the next reporting period to accomplish the goals?**

  I expect that the most significant and impactful outcomes of this grant will be produced in year two, largely due to the time taken to obtain the IACUC and ACURO approval, and also due to the challenges I had during the first year.

  - Finish Major Task 1 anticipate to receive ACURO approval in November.
  - Finish Major Task 2 DLD-1, HCT116, HT29, SW620, Caco-2, CT26, and MC28 (if found).
  - Start and finish Major Task 3 once ACURO approval is received.
  - Start and finish Major Task 4 once ACURO approval is received.
  - Start and finish Major Task 5 publish Manuscript.

IMPACT:

- **What was the impact on the development of the principal discipline(s) of the project?**
  - Nothing to Report.
- **What was the impact on other disciplines?**
  - Nothing to Report.
- **What was the impact on technology transfer?**
  - Nothing to Report.
- **What was the impact on society beyond science and technology?**
  - Nothing to Report

CHANGES/PROBLEMS:

- **Changes in approach and reasons for change**
  - Cell lines appear to be responding differently based on my initial experiments. Before proceeding with PK/animal experiments the mechanism of action of CBD on CRC cells needs to be further investigated. The fact that these cell lines appear to be responding differently may indicate that there are potential sub-classes of CRC that are more sensitive to CBD. This has also shown to be different then in the few reported literature articles on these cell lines and CBD, and should be explored further before proceeding with any mice studies.
  - The university has set up a mentorship committee consisting of three Biology faculty and one Agriculture faculty to guide successful completion of my grant work.
- **Actual or anticipated problems or delays and actions or plans to resolve them**
  - The number one delay that this project has encountered is due to the Covid-19 pandemic. This has kept regulatory bodies from meeting and has been unclear due to my hi risk status due to my MS as to how I was to respond in March and April.
  - The second issue has been my health. The pandemic increased my stress levels. I had a surgery in April, hospitalization due to high blood pressure in June, adverse medication interactions, seizures, and tachycardia in July.

- As I transferred to MTSU, the orientation, safety training, procurement training, getting familiar with the new lab and facility took about a month.

- I continue to move forward despite these issues even though at a slower pace. I have been working with my mentorship committee to develop a solid project plan to move the project forward.

- **Changes that had a significant impact on expenditures**

  - The cost of mice has increased by approximately $5,000 ($25 each for C57BL/6 mice to $76) due to the pandemic. However, MTSU College of Basic and Applied Sciences has committed to cover the additional cost.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

  - Nothing to Report

- **Significant changes in use or care of human subjects**

  - Nothing to Report

- **Significant changes in use or care of vertebrate animals.**

  - Nothing to Report

- **Significant changes in use of biohazards and/or select agents**

  - Nothing to Report

PRODUCTS:

- **Publications, conference papers, and presentations**

  - Nothing to report

- **Website(s) or other Internet site(s)**

  - Nothing to Report

- **Technologies or techniques**

  - Nothing to Report

- **Inventions, patent applications, and/or licenses**

  - Nothing to Report

- **Other Products**

  - Nothing to Report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name:	*Gina Bishara*
Project Role:	*Undergraduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Ms. Bishara has performed work in the area of cell culture and assisted in cell treatments of colon cancer lines with the CBD formulations*
Funding Support:	NA

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
    - Nothing to Report.
- **What other organizations were involved as partners?**
    - Nothing to Report

SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:** *For collaborative awards, independent reports are required from **BOTH** the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to https://ebrap.org for each unique award.*
- **QUAD CHARTS:** *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Reminder: Pages shall be consecutively numbered throughout the report. **DO NOT RENUMBER PAGES IN THE APPENDICES.***