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In vitro antioxidant and anticancer activities of *Clinacanthus nutans* extracts

Joyce Hui Yie Phung[1], Isabel Lim Fong[1], Heng Yen Khong[2] and Weng Kit Ban[1]*

[1] Faculty of Medicine and Health Sciences, Universiti Malaysia Sarawak, Kota Samarahan 94300, Sarawak, Malaysia
[2] Faculty of Applied Sciences, Universiti Teknologi MARA, Kota Samarahan 94300, Sarawak, Malaysia

***Corresponding author**:
Isabel Lim Fong
flisabel@unimas.my

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ABSTRACT

Clinacanthus nutans (CN), from the Acanthaceae family is a medicinal plant widely used in Thailand and Malaysia. CN is commonly used as a treatment of inflammation, cancer, and herpes virus infection. This study aimed to determine the antioxidant activity and anticancer properties of CN leaves extracts on human colorectal cancer cell lines, HCT 116 and HT-29. In this study, CN leaves powders were extracted in methanol, chloroform, and acetone at different durations. The crude extracts were assessed for total phenolic content (TPC), total flavonoid contents (TFC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay. The extract using acetone showed with the highest TPC. The highest total flavonoid content was the methanol extract. The DPPH radical scavenging activity, IC_{50} of methanol extract was 19.67 μg/mL and exhibited the most efficacious antioxidant property among the others. The 24-hour methanol extract showed the most promising results on MTT assay. Therefore, CN methanol extract is a promising candidate to proceed in other anticancer studies such as cell cycle arrest and apoptosis assays.

Keywords: *Clinacanthus nutans*; colorectal cancer; anticancer; antioxidants; phenolic; flavonoid

1. INTRODUCTION

Cancer causes the second highest mortality in the world. In 2018, it was estimated to result in 9.6 million deaths (WHO, 2019). Colorectal cancer is the third most fatal cancer and the fourth most diagnosed cancer worldwide (Rawla et al., 2019). According to the Malaysian national cancer registry report 2012-2016, colorectal cancer was the most common cancer in male and the second most common in female (Azizah et al., 2019). It is possible to prevent colorectal cancer progression if early polyps were detected. Nonetheless, early detection of polyps is not routinely performed unless the patient has underlying hereditary history of colorectal cancer. This is commonly treated with surgery and in the advanced stage, a single chemotherapeutic drug, 5-fluorouracil (5-FU) would be routinely used or combined with adjuvants like avastin and oxaliplatin (Sheikh et al., 2017a; Cutsem et al., 2015). Undetected polyps usually lead to formation of adenomas and carcinomas before the late stage of metastasis leading to high mortality risk (Sheikh et al., 2017a).

Many studies have reported that increased levels of intracellular free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) triggered cancer formation (Yong et al., 2013). ROS and RNS are normally found in the human body as they are by-products of metabolism in normal cells (Yong et al., 2013). These ROS and RNS damage the normal cells, especially DNA, resulting in genomic instability and induce cancer initiation (Yong et al., 2013). Many medicinal plants have been reported to

contain plethora of natural antioxidant compounds that can eliminate ROS and RNS in the human body (Sheikh et al., 2017a). Moreover, bioactive compounds found in plants and herbs have been widely screened and studied in pre-clinical and clinical settings as they are reported to have anticancer properties including inhibition of cancer growth, cell cycle arrest and induction of apoptosis (Sheikh et al., 2017a; Sheikh et al., 2017b; Zlotogorski et al., 2013; Schaffer et al., 2015; Tungmunnithum et al., 2018). Subsequently, it is pertinent and timely to bioprospect traditional herbal remedies and plants as alternative cancer treatment.

Clinacanthus nutans (CN) is a medicinal plant from Acanthaceae family and is commonly used in Southeast Asia especially Malaysia and Thailand as home remedies for various purposes (Yong et al., 2013; Zulkipli et al., 2017). Zulkipli (2017) reported the different traditional therapeutic uses of CN including the most common uses of CN as anti-venom, herpes virus infection treatment, and cancer treatment (Zulkipli et al., 2017). There are many products of CN found in the market such as leaves powder, creams, capsules, and ointments (Zulkipli et al., 2017). Although there are several studies on the antioxidant and anticancer properties of CN on various types of cancers, there are very few studies on the comparison of different polarity solvents and duration to effectively extract specific CN compounds. Furthermore, reports on the suppression of CN extracts on colorectal cancer cell lines in dose- and time-dependent manner are very limited. Therefore, this study aimed to determine the antioxidant activity and anticancer properties of extracts on human colorectal cancer cell lines, HCT 116 and HT-29 and normal human cell line NP69 in relation to the different polarities of solvents and duration of extraction on CN leaves.

2. MATERIALS AND METHODS

2.1 Preparation of CN extracts

CN was harvested as whole plant from Stapok, Kuching, Sarawak in November 2018. The leaves were plucked, washed, and dried at a room temperature before being ground into powder form. These powders (50 g) were soaked in methanol, chloroform, or acetone, at a ratio of 1:10 CN powdered leaves: solvent. Extraction duration of 30 minutes was carried out to determine the antioxidant activity and anticancer properties of CN leaves by using minimum extraction time and compared with 24 hours. The CN powders were then continuously stirred in solvents for 30 minutes and 24 hours by using a magnetic stirrer (FAVORIT Magnetic Stirrer, Puchong, Malaysia). The crude extracts were filtered through filter paper (Whatman® quantitative filter paper, ashless, Grade 42) and left to dry in a fume hood. The crude extracts were stored in the dark at 4°C for further use.

2.2 Total phenolic content assay

The total phenolic content (TPC) of CN extracts was determined by using Folin-Ciocalteu reagent method according to Baba and Malik (2015) with slight. Firstly, 200 μL of 1 mg/mL CN extracts were made up to 3 mL with distilled water and mixed with 500 μL of Folin-Ciocalteu reagent (diluted half fold with distilled water) for 3 minutes. Once mixed well, 2 mL of 20% w/v sodium carbonate solutions were added and incubated for an hour in the dark. The absorbance was measured at wavelength 650 nm by using a spectrophotometer (Thermo Scientific GENESYS 10, Waltham, USA). Gallic acid (500 μg/mL) was used as a standard reference and was serially diluted using ethanol to establish a calibration curve. The TPC of CN extracts was expressed in the unit of mg/g, gallic acid equivalents per dry weight of CN extracts.

2.3 Total flavonoid content assay

The total flavonoid content (TFC) of CN extracts was determined by using aluminum chloride colorimetric method adapted from Chan et al. (2012). The 1 mg/mL CN crude extracts was serial diluted in methanol before 1 mL of the diluted extracts was mixed with 1 mL of 2% aluminum chloride. These were incubated for 10 minutes and the absorbance was measured at wavelength 420 nm by using a spectrophotometer (Thermo Scientific GENESYS 20, Waltham, USA). Methanol solvent was used as a blank. A serial dilution of quercetin dihydrate (100 μg/mL) was prepared and used as the standard to establish the calibration curve. The TFC of CN extracts was expressed in the unit of mg/g, quercetin dihydrate equivalents per dry weight of CN extracts.

2.4 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) Radical scavenging assay

To assess the antioxidant potential of the CN extracts, 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay was conducted according to Herald et al. (2012) with slight modification. Firstly, 1 mL of CN extracts at different concentrations between 10 and 100 μg/mL was mixed with 1 mL of 100 μM DPPH. The mixture was incubated for 30 minutes in the dark. The absorbance was measured at wavelength 517 nm using a spectrophotometer (Thermo Scientific GENESYS 20, Waltham, USA). Ascorbic acid was used as a standard. In this method, antioxidant activity was described by scavenging ability (%):

$$\text{DPPH free radical scavenging activity (\%)} = [(A_0 - A_S) / A_0] \times 100 \quad (1)$$

where A_0 represents the absorbance value of control (without sample) and A_S represents the absorbance value of the sample.

2.5 *In vitro* assays

2.5.1 Cell culture

Two human colorectal cancer cell lines of different progression stages were selected for these assays. These were human colorectal carcinoma cell line (HCT 116) and human colorectal adenocarcinoma cell line (HT-29). A normal human epithelial cell line (NP69) was used as a control. HCT 116 and HT-29 were cultured in McCoy's 5A (modified) medium with 10% fetal bovine serum whereas NP69 was cultured in keratinocyte serum-free medium with 2% fetal bovine serum, 25 mg bovine pituitary extract, and 2.5 µg epidermal growth factor, human recombinant. Both media were filtered through a 0.22-µm filter membrane (Minisart, Sartorius Stedim, Goettingen, Germany). The cells were incubated at 37°C, humidified with 5% CO_2. These were sub-cultured every three to four days once they had reached 80% confluency. The cells were detached by using 0.05% trypsin-EDTA and cell counting was performed to obtain the desired cell number using trypan blue and a haemocytometer. CN extracts and positive control cisplatin were used in this study whereas

untreated cells with media only or media with 0.1% dimethyl sulfoxide (DMSO) were used as control.

2.5.2 Antiproliferation assay to assess *Clinacanthus nutans* extracts using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

To assess the ability of these CN extracts in triggering antiproliferation of HCT 116, HT-29 and NP69, MTT reduction assay was performed according to Sheikh et al. (2017b) with slight modification. MTT assay was used to determine cell viability based on the activity of mitochondrial enzymes, for example, succinate dehydrogenase. Its activity would generate purple insoluble formazan in the media, an indication of viable cells (Stone et al., 2009).

Firstly, 5000 viable HCT 116 cells, 10,000 viable HT-29 cells and 1000 viable NP69 cells were seeded in 96-well plates for 24 hours. The culture media was then replaced by CN methanol extracts of two different durations, 30 minutes and 24 hours, between 7.8 and 250 μg/mL. The positive control cisplatin, was also administered at concentrations between 0.9 and 30 μg/mL. The treated cells were incubated for 24, 48, and 72 hours at 37°C in a 5% CO_2, humidified incubator. After incubation, the extracts and cisplatin were replaced with 200-μL fresh culture media and 20 μL of 5 mg/mL MTT reagent. These cells were incubated in the dark for 4 hours at 37°C in a 5% CO_2, humidified incubator before the formazan crystals were solubilized with 150-μL DMSO. The absorbance was measured at wavelength 570 nm using a microplate reader (SpectraMax iD3 Multi-Mode Microplate Reader, Molecular Devices, San Jose, California, USA). The cell viability was calculated according to the formula:

$$\text{Cell viability (\%)} = \frac{\text{Absorbance at 570nm of sample}}{\text{Absorbance at 570nm of control}} \times 100\ \% \quad (2)$$

2.6 Statistical analysis

All experiments were conducted in triplicates. All data were caulcuated and expressed as mean ± standard error using IBM® SPSS® Version26, USA.

3. RESULTS AND DISCUSSION

3.1 TPC of CN extracts

The TPC of CN leaves extracts was determined by using Folin-Ciocalteu reagent method. The results showed that the acetone extracts with 30 minutes and 24 hours extraction time gave the highest TPC at 4.24 mg/g and 4.33 mg/g, respectively (Figure 1). Among all the extracts, the methanol extract with extraction durations of 30 minutes and 24 hours had the lowest TPC of 2.08 mg/g and 2.04 mg/g, respectively.

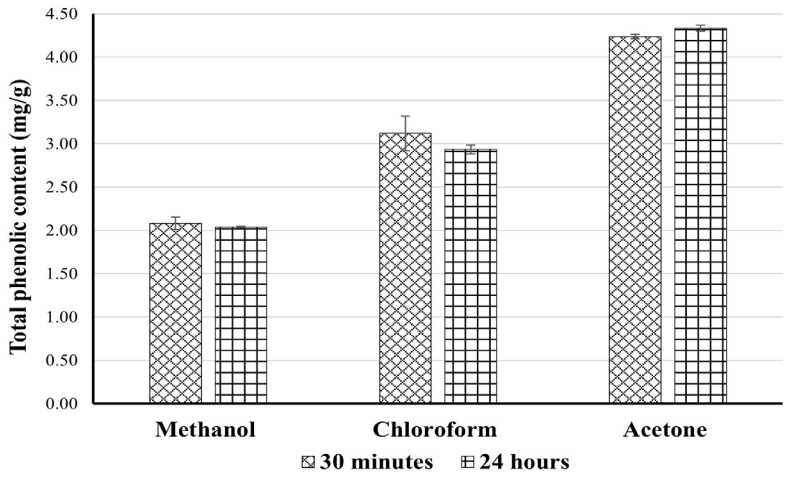

Figure 1. Total phenolic content in CN extracts with different durations of extraction
Note: TPC was expressed in the unit of mg/g, gallic acid equivalents per dry weight of CN extracts. Results represent mean ± standard deviation (n=3)

3.2 TFC of CN extracts

The TFC of CN leaves extracts was assessed by using aluminum chloride colourimetric method. The results indicated that the methanol extracts at extraction duration of 30 minutes and 24 hours showed the highest TFC at 0.47 mg/g and 0.48 mg/g, respectively (Figure 2).

3.3 Antioxidant activity

The antioxidant activity of CN extracts was determined by DPPH radical scavenging assay. The results of the scavenging ability, IC_{50} of CN extracts were summarized in Table 1. Chloroform extracts showed the lowest antioxidant activities with the highest IC_{50} value, compared to other extracts, which was 92.46 μg/mL at 30 minutes extraction duration and 40.25 μg/mL at 24 hours extraction duration. The methanol extracts demonstrated the highest antioxidant activities with the lowest IC_{50} values. Ascorbic acid was used as a positive control and its the IC_{50} was 5.37 $\pm$ 0.17 μg/mL.

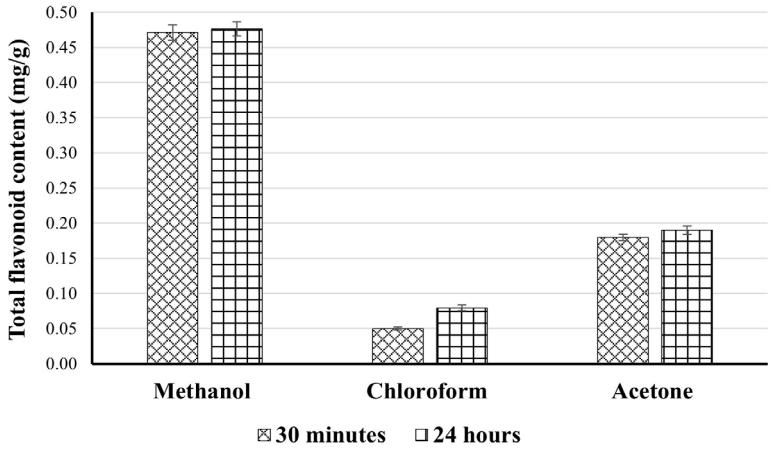

Figure 2. Total flavonoid content in CN extracts with different durations of extraction
Note: TFC was expressed in the unit of mg/g, quercetin dihydrate equivalents per dry weight of CN extracts. Results represent mean ± standard deviation (n=3)

Table 1. DPPH free radical scavenging activity of CN methanol, chloroform and acetone extracts with 30 minutes and 24 hours extraction time

Solvents	IC50 (μg/mL)	
	30 minutes	24 hours
Methanol	24.25 ± 0.75	19.67 ± 0.62
Chloroform	92.46 ± 9.45	40.25 ± 6.15
Acetone	32.25 ± 0.20	23.17 ± 1.55

Note: The scavenging activity was expressed as IC_{50}
Results represent mean ± standard deviation (n=3)

3.4 Von Mises stress of the implant

The antiproliferation activity of CN methanol extracts was evaluated by MTT assay. The results of the IC_{50} of CN methanol extracts at different extraction durations of 30 minutes and 24 hours and cisplatin on HCT 116, HT-29 and NP69 are shown in Table 2. The IC_{50} of CN methanol extracts at 30 minutes extraction duration was 58.28 μg/mL on HCT 116 after 72 hours of treatment. On the other hand, CN methanol extracts at 24 hours extraction duration showed IC_{50} of 239 μg/mL and 72.37 μg/mL on HCT 116 after 48 hours and 72 hours of treatment, respectively. CN methanol extracts at 24 hours extraction duration also showed IC_{50} of 182.67 μg/mL after 48 hours of treatment on HT-29. Nevertheless, CN methanol extracts at 24 hours extraction duration resulted in IC_{50} of 55.60 μg/mL after 72 hours of treatment on NP69. The chemotherapy drug, cisplatin showed significance cytotoxic effect on all the cell lines.

Table 2. IC_{50} (μg/mL) of CN methanol extracts (30 minutes and 24 hours) and cisplatin on HCT 116, HT-29 and NP69

	HCT 116	HT-29	NP69
CN methanol extract (30 minutes)			
24 hours	> 250	> 250	NA
48 hours	> 250	> 250	NA
72 hours	58.28 ± 24.24	NA	> 250
CN methanol extract (24 hours)			
24 hours	> 250	> 250	NA
48 hours	239 ± 4.24	182.67 ± 45.07	NA
72 hours	72.37 ± 20.44	NA	55.60 ± 13.78
Cisplatin			
24 hours	18.98 ± 9.56	6.76 ± 0.77	NA
48 hours	4.74 ± 0.08	3.90 ± 0.61	NA
72 hours	2.63 ± 0.91	NA	1.31 ± 0.96

Note: NA - no activity; results represent mean ± standard deviation (n=3)

4. DISCUSSION

ROS and RNS are ubiquitously produced by our bodies. However, an unbalanced level of ROS and RNS induces oxidative stress that can cause DNA instability, leading to cancer. Phenolic compounds and flavonoids are plant secondary metabolites, which are usually produced by plants to protect themselves from oxidation and support their growth under stress conditions (Tungmunnithum et al., 2018). Medicinal plants such as CN were reported that they contain natural antioxidant compounds, especially phenolic compounds and flavonoids. In this study, CN acetone extracts had the highest level of phenolic compounds. According to Galanakis et al. (2013), natural phenolic compounds preferred semi-polar solvents such as acetone and alcohol rather than higher polar and lower polar or non-polar

solvents (Galanakis et al., 2013). However, CN methanol extracts had the lowest content of phenolic compounds in this study. In fact, it was difficult to select a suitable solvent for extraction as it requires a complex procedure as the efficiency is dictated by a complex interaction of parameters (Galanakis et al., 2013). On the other hand, CN methanol extracts possessed the highest total flavonoid content than those of acetone and chloroform although it showed the lowest total phenolic contents. This may due to the phenolic compounds in CN methanol extracts contained most of the flavonoids, whereas CN acetone extracts contained fewer flavonoids than other phenolic compounds.

The antioxidant activity of CN was determined through DPPH radical scavenging activity assay. DPPH solution reacts with antioxidant compounds that can donate hydrogen to DPPH free radicals (Ksouri et al., 2007). The reduction of DPPH free radicals causes a change of color from purple to yellow. CN methanol extracts showed the lowest IC_{50} of DPPH free radicals. The lower the IC_{50}, the more efficient to scavenge oxidants like DPPH free radicals. Although CN methanol extracts had the lowest TPC, it had the highest antioxidant activity. Generally, if the antioxidant activity is higher, it is expected to have high phenolic and flavonoid contents. Similarly, Sengul et al. (2009) reported that there is no relationship between TPC and antioxidant activity. Moreover, Nickavar et al. (2007) elucidate that only flavonoids with a hydroxyl group enhance free radical scavenging activity due to proton donation. This was evident as that CN methanol extracts with higher TFC showed the highest antioxidant activity.

Besides that, the results of TPC, TFC, and antioxidant activity of different extraction durations showed not much difference in their contents and activities. However, 24 hours of extraction duration showed slightly higher TPC in CN acetone extracts and TFC of all *CN* extracts. Extraction duration of 24 hours also showed lower IC_{50} than those of 30 minutes in all CN extracts.

Based on the results of TPC, TFC and antioxidant activity, CN methanol extracts of 30 minutes and 24 hours extraction durations were the most promising candidates to assess the antiproliferation study of human colorectal cancer cell lines, HCT 116, and HT-29 and normal human epithelial cells (NP69). In this study, both CN methanol extracts showed a reduction in proliferation in HCT 116 and NP69 after 72 hours of treatment. Similarly, CN methanol extract of 24 hours extraction duration showed reduction in viable cells in HCT 116 and HT-29 after 48 hours of treatment. This study showed that CN methanol extract of 24 hours extraction duration had lower IC_{50} than that of 30 minutes extraction duration. According to Wang et al. (2019), CN ethyl acetate fraction exhibited the lowest IC_{50} (48.81 μg/mL) on HCT 116. Ethyl acetate, a semi-polar solvent, proved that semi-polar solvent results in a better cytotoxic effect on HCT 116 than polar and non-polar solvents as it induced ROS-dependent apoptosis and autophagy (Wang et al., 2019). However, Quah et al. (2017) reported that methanol extracted CN for 48 hours repeatedly through maceration method, resulted in lower IC_{50} (20.72 μg/mL) on HT-29. This indicated that the longer extraction duration might exhibit more effective antiproliferation effect on HT-29. The study reported that CN methanol extract contained flavonoids such as orientin and vitexin by HPLC-IT/MS analysis (Quah et al., 2017). Other studies had been done to demonstrate that orientin and vitexin induce apoptosis in HT-29 and HCT 116 (Thangaraj et al., 2019; Bhardwaj et al., 2018). The assay would be repeated with a higher concentration of both CN methanol extracts as there was no IC_{50} shown within the concentration range, especially at 24 hours treatment. The assay should be repeated with a higher number of NP69 cell to achieve a more significant IC_{50}. Overall, the results showed that CN methanol extracts, especially with 24 hours extraction duration, possessed potential antiproliferation ability against colorectal cancer cells, HCT 116 and HT-29.

5. CONCLUSION

In conclusion, methanol was the suitable solvent to extract CN leaves as CN methanol extracts showed the highest TFC and antioxidant activity than chloroform and acetone extracts. Furthermore, 24-hour extraction duration was effective to extract CN leaves as it resulted in lower IC_{50} of antioxidant and antiproliferation activities in HCT 116 and HT-29. Besides that, 30 minutes of extraction duration was considered sufficient to extract CN leaves as it showed similar TPC and TFC with 24 hours extraction duration. As such, CN methanol extract at 24 hours extraction duration was a promising candidate to proceed in other anticancer studies such as cell cycle arrest and apoptosis assays. MTT assay should be repeated with higher concentration of CN methanol extracts and higher NP69 cell number.

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REFERENCES

Azizah, A. M., Hashimah, B., Nirmal, K., Siti Zubaidah, A. R., Puteri, N. A., Nabihah, A., Sukumaran, R., Balqis, B., Nadia, S. M. R., Sharifah, S. S. S., Rahayu, O., Nur Alham, O., and Azlina, A. A. (2019). *Malaysia National Cancer Registry Report 2012-2016*. Putrajaya, Malaysia: National Cancer Registry, pp. 1-116.

Baba, S. A., and Malik, S. A. (2015). Determination of total phenolic and flavonoid content, antimicrobial and antioxidant activity of a root extract of *Arisaema jacquemontii* Blume. *Journal of Taibah University for Science*, 9(4), 449-454.

Bhardwaj, M., Cho, H., Paul, J. S., Jakhar, R., Khan, I., Lee, S. J., Kim, B. Y., Krishnan, M., Khaket, T. P., Lee, H. G., and Kang, S. C. (2018). Vitexin induces apoptosis by suppressing autophagy in multi-drug resistant colorectal cancer cells. *Oncotarget*, 9(3), 3278-3291.

Chan, K. W., Khong, N. M., Iqbal, S., and Ismail, M. (2012). Simulated gastrointestinal pH condition improves antioxidant properties of wheat and rice flours. *International Journal of Molecular Sciences*, 13(6), 7496-7507.

Cutsem, E. V., Lenz, H. J., Kohne, C. H., Heinemann, V., Tejpar, S., Melezínek, I., and Ciardiello, F. (2015). Fluorouracil,

leucovorin, and irinotecan plus cetuximab treatment and RAS mutations in colorectal cancer. *Journal of Clinical Oncology*, 33(7), 692-700.

Galanakis, C. M., Goulas, V., Tsakona, S., Manganaris, G. A., and Gekas, V. (2013). A knowledge base for the recovery of natural phenols with different solvents. *International Journal of Food Properties*, 16(2), 382-396.

Herald, T. J., Gadgil, P., and Tilley, M. (2012). High-throughput micro plate assays for screening flavonoid content and DPPH-scavenging activity in sorghum bran and flour. *Journal of the Science of Food and Agriculture*, 92(11), 2326-2331.

Ksouri, R., Megdiche, W., Debez, A., Falle, H., Grignin, C., and Abdelly, C. (2007). Salinity effects of polyphenol content and antioxidant activities in leaves of the halophyte *Cakile maritimai. Plant Physiology and Biochemistry*, 45(3-4), 244-249.

Nickavar, B., Kamalinejad, M., and Izadpanah, H. (2007). In vitro free radical scavenging activity of five Salvia species. *Pakistan Journal of Pharmaceutical Sciences*, 20(4), 291-294.

Quah, S. Y., Chin, J. H., Akowuah, G. A., Khalivulla, S. I., Yeong, S. W., and Sabu, M. C. (2017). Cytotoxicity and cytochrome P450 inhibitory activities of Clinacanthus nutans. *Drug Metabolism and Personalized Therapy*, 32(1), 59-65.

Rawla, P., Sunkara, T., and Barsouk, A. (2019). Epidemiology of colorectal cancer: Incidence, mortality, survival, and risk factors. *Przegląd Gastroenterologiczny*, 14(2), 1-15.

Schaffer, M., Schaffer, P. M., and Bar-Sela, G. (2015). An update on Curcuma as a functional food in the control of cancer and inflammation. *Current Opinion in Clinical Nutrition and Metabolic Care*, 18(6), 605-611.

Sengul, M., Yildiz, H., Gungor, N., Cetin, B., Eser, Z., and Ercisli, S. (2009). Total phenolic content, antioxidant, and antimicrobial activities of some medicinal plants. *Pakistan Journal of Pharmaceutical Sciences*, 22(1), 102-106.

Sheikh, B. Y., Sarker, M. M. R., Kamarudin, M. N. A., and Mohan, G. (2017a). Antiproliferative and apoptosis inducing effects of citral via p53 and ROS-induced mitochondrial-mediated apoptosis in human colorectal HCT116 and HT29 cell lines. *Biomedicine and Pharmacotherapy*, 96, 834-846.

Sheikh, B. Y., Sarker, M. M. R., Kamarudin, M. N. A., and Ismail, A. (2017b). Prophetic medicine as potential functional food elements in the intervention of cancer: a review. *Biomedicine and Pharmacotherapy*, 95, 614-648.

Stone, V., Johnston, H., and Schins, R. P. (2009). Development of *in vitro* systems for nanotoxicology: methodological considerations. *Critical Reviews in Toxicology*, 39(7), 613-626.

Thangaraj, K., Balasubramanian, B., Park, S., Natesan, K., Liu W., and Manju, V. (2019). Orientin induces G0/G1 cell cycle arrest and mitochondria mediated intrinsic apoptosis in human colorectal carcinoma HT29 Cells. *Biomolecules*, 9(9), 418.

Tungmunnithum, D., Thongboonyou, A., Pholboon, A., and Yangsabai, A. (2018). Flavonoids and other phenolic compounds from medicinal plants for pharmaceutical and medical aspects: An overview. *Medicines*, 5(3), 93.

Wang, K. S., Chan, C. K., Hidayat, A. F. A., Wong, Y. H., and Kadir, H. A. (2019). *Clinacanthus nutans* induced reactive oxygen species-dependent apoptosis and autophagy in HCT116 human colorectal cancer cells. *Pharmacognosy Magazine*, 15(60), 87.

WHO. (2019). Cancer. *World Health Organization*. [Online URL: https://www.who.int/news-room/fact-sheets/detail/cancer] accessed on November 22, 2020.

Yong, Y. K., Tan, J. J., Teh, S. S., Mah, S. H., Ee, G. C. L., Chiong, H. S., and Ahmad, Z. (2013). *Clinacanthus nutans* extracts are antioxidant with antiproliferative effect on cultured human cancer cell lines. *Evidence-Based Complementary and Alternative Medicine*, 2013, 462751.

Zlotogorski, A., Dayan, A., Dayan, D., Chaushu, G., Salo, T., and Vered, M. (2013). Nutraceuticals as new treatment approaches for oral cancer–I: curcumin. *Oral Oncology*, 49(3), 187-191.

Zulkipli, I. N., Rajabalaya, R., Idris, A., Sulaiman, N. A., and David, S. R. (2017). *Clinacanthus nutans*: a review on ethnomedicinal uses, chemical constituents and pharmacological properties. *Pharmaceutical biology*, 55(1), 1093-1113.