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Original Research Article

Chemopreventive properties of curcumin analogues, hexagamavunone-0 and gamavutone-0, in rat colorectal cancer model

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Abstract

Purpose: *To examine the chemopreventive activity of curcumin analogues, hexagamavunone-0 (HGV-0) and gamavutone-0 (GVT-0), compared to curcumin in a colorectal cancer model in Wistar rats.*
Methods: *Rats (n = 25) were assigned to one of five groups (n = 5 in each group). Colorectal cancer was induced in the control group with subcutaneous injection of 1,2-dimethylhydrazine (DMH) 60 mg/kg once a week for 15 weeks. In addition to DMH injection, treatment groups were treated with curcumin (20, 40, or 80 mg/kg), gamavutone-0 (GVT-0; 20, 40, or 80 mg/kg), and hexagamavunone (HGV-0; 20, 40, or 80 mg/kg) orally twice a week for 15 weeks. The number and volume of nodules in the colorectal area were observed after laparatomy. Histopathological analysis was performed using H & E staining and immunohistochemistry with antibodies against adenomatous polyposis coli (APC) and cyclooxygenase 2 (COX-2).*
Results: *All treatments reduced colorectal nodule volume, but only HGV-0 significantly decreased the numbers of nodules compared to DMH controls ($p < 0.05$). The reduction was 96.1 % with 40 mg/kg HGV-0. Mutated APC expression was inhibited by curcumin, GVT-0, and HGV-0 at a dose of 40 mg/kg, whereas COX-2 expression was mostly inhibited by HGV-0 (20 and 40 mg/kg) and curcumin to a lesser extent, but not inhibited by GVT-0 treatment in rat colorectal cancer.*
Conclusion: *HGV-0 showed superior chemoprevention compared to GVT-0 and curcumin. HGV-0 at a dose of 40 mg/kg significantly reduced the number and volume of colorectal nodules. The mechanism of chemoprevention of HGV-0 is related to its inhibition of APC mutation and COX-2 expression.*

Keywords: *Curcumin, Gamavutone-0, Hexagamavunone-0, Colorectal cancer, Adenomatous polyposis coli, Cyclooxygenase-2*

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INTRODUCTION

Colorectal cancer is the fourth most commonly diagnosed malignancy in developed countries and the fifth in less developed countries [1]. Several factors have been shown to induce colorectal cancer growth, including heredity, gene mutations, diet, and lifestyle [2]. In addition, the carcinogenic process in colorectal cancer may involve epigenetic alterations, including alteration of DNA methylation, histone degradation, and mRNA expression [3,4].

One strategy to delay the carcinogenesis process is with the use of chemopreventive agents. Curcumin is a plant-derived

chemopreventive agent that is currently in phase II clinical trials [5,6]. It is found in the dried rhizome of *Curcuma longa* Linn, a common herb used in Asia, including India, China, and Indonesia. Since 1995, many studies have demonstrated the efficacy of curcumin to inhibit or reduce the growth of colorectal cancers [6-8]. Furthermore, a range of biological activities have been proposed for curcumin extract, including antioxidant, anti-inflammatory, chemopreventive, and chemotherapeutic effects [9,10]. With respect to its chemopreventive properties, the mechanisms of action of curcumin are associated with the inhibition of cyclooxygenase 2 (COX-2), tumor necrosis factor alpha (TNF-a), and nuclear factor kappa B (NF-kB) as well as the attenuation of cancer-cell proliferation [5,10,11].

Two analogues of curcumin, hexagamavunone (HGV-0) and gamavutone-0 (GVT-0), were synthesized in 1954 using the Rumpel method with some modifications [12]. These compounds are reported to have antitumor activities [13]. In addition, HGV-0 possesses anti-inflammatory activity through its inhibition of COX enzyme activity [12,13], with IC_{50} values of 8.15 μM and 8.02 μM [12].

The other curcumin analogue, GVT-0, also has antioxidant, anti-inflammatory, antitumor, antibacterial, and antifungal activities [12]. Another report shows that GVT-0 has a potent anti-proliferative effect because it inhibits endothelial cell proliferation by 96.6 % at a concentration of 3 μg/mL [14]. Indeed, the antioxidant activity of GVT-0 is more potent than that of curcumin [15].

The aim of the present study was to examine the chemopreventive activity of curcumin analogues (GVT-0 and HGV-0) on a colorectal cancer model induced by 1,2-dimethylhydrazine (DMH) in Wistar rats.

EXPERIMENTAL

Materials

Curcumin was obtained from Merck (Germany), curcumin analogues (GVT-0 and HGV-0) and a rabbit polyclonal antibody against adenomatous polyposis coli (APC), were obtained from Thermo Fisher Scientific (Waltham, MA, USA), COX-2 rabbit polyclonal antibody was obtained from Lab Vision Corp (Fremont, CA, USA), biotinylated anti-mouse and rabbit secondary antibodies were obtained from Biocare Medical (USA), and 1,2-dimethylhidrazine.2HCl (DMH) was obtained from ABCR (Germany).

Animal preparation

Twenty five male Wistar rats (150 – 200 g) were obtained from Gadjah Mada University animal house. The rats were acclimatized in Multidisciplinary Research an Analysis Laboratory of Gadjah Mada University at least two weeks prior to experiment. The rats were cared in environmentally controlled condition with 12-hour light and dark cycle in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals [16], and allowed to feed on standard rodent pellets and water ad libitum. The experimental protocol for animal use for this study is approved by the Ethics Committee of Gadjah Mada University Indonesia with approval no. KE/FK/08/EC.

Induction of colorectal carcinoma and treatment with GVT-0 and HGV-0

Male Wistar rats aged 6 weeks (n = 25) were assigned to one of five treatment groups. The control group was subcutaneously injected with DMH at a dose of 60 mg/kg body weight (BW) once a week [15] and given 0.5 % sodium carboxyl methyl cellulose (Na CMC) orally twice a week for a total of 15 weeks. Treatment groups were subjected to DMH injection once a week followed by curcumin (20, 40, or 80 mg/kg BW), GVT-0 (20, 40, or 80 mg/kg BW), or HGV-0 (20, 40, or 80 mg/kg BW) administration via oral gavage twice a week for 15 weeks. Animals were sacrificed one week after all treatments were completed.

Macroscopic observation

A laparotomy was performed through the main arch to gain access to the abdominal cavity. The intestines were harvested and washed with distilled water. The ileum, colorectal, rectum, and small bowel were placed on a polystyrene board and cut open with the intestinal mucosa facing upwards to observe the presence of neoplasms, and the number and volume of nodules were recorded for analysis [17]. Calculation of nodule volume is presented in Eq 1.

$$VN\ (mm^3) = \{NL(NW)^2 \times 0.52\} \quad \text{..... (1)}$$

where VN = volume of nodules, NL = nodule length, NW = nodule width

Histopathological examination

Paraffin-embedded tissues were cut using a microtome (Leica Biosystems) to a thickness of 5 μm. Slides were de-waxed with xylol followed by step-wise rehydration (100, 95 and 70 %

ethanol). Slides were then rinsed with water for 1 minute and stained with hematoxylin-eosin (HE).

Immunohistochemistry

Paraffin-embedded tissues were cut using a microtome (Leica Biosystems) to a thickness of 5 μm and placed on poly-L-lysine object glass (Muto Pure Chemicals Co., Ltd., Japan). Slides were de-waxed and subsequently rehydrated with 100, 95 and 70 % ethanol. The slides were treated with one drop of 3 % H_2O_2 in aquadest and allowed to stand for 30 min to block endogenous peroxidase in the tissues. They were then incubated for 60 min at room temperature with COX-2 (Lab Vision Corp.) and APC primary antibodies (Thermo Fisher Scientific). This procedure was undertaken to observe COX-2 and APC-mutated expression. APC and COX-2 expression were calculated using the immunoreactivity (IMR) as in Eq 2 [13].

IMR (%) = {(CCp/CCt)100 …… (2)

where IMR is immunoreactivity, CCp is cell count with APC or COX-2 positive staining, and CCt the sum of positive and negative cell counts.

Statistical analysis

All data were presented as mean ± SEM and analyzed using SPSS software version 20. Parametric data was analyzed with one-way ANOVA followed by post-hoc test. Positive staining was visualized as the presence of brown color in the cytoplasm. $P < 0.05$ was taken to indicate statistically significant difference.

RESULTS

Macroscopic features

Subcutaneous injection of DMH at a dose of 60 mg/kg BW once a week, for a total of 15 weeks induced colorectal nodules, with average of 5.2 ± 0.92 nodules per rat in the control group. Treatment with curcumin twice a week reduced the presence of nodules, with an observed reduction of as much as 61.5 % in the group treated with 40 mg/kg BW dose compared to controls (Figure 1). However, this number was not statistically significant due to the large standard error of the mean (SEM) in the control group. Meanwhile, treatment with HGV-0 twice a week significantly reduced nodule appearance in the colon area. HGV-0 at a dose of 20 mg/kg BW significantly reduced the number of nodules, but the optimal dose was 40 mg/kg BW, which led to a reduction of up to 96.1 % in nodule numbers ($p < 0.05$). In GVT-0 treated rats, the presence of nodules in colorectal cancer decreased to as low as 3.2 ± 1.02 nodules per rat, but the change was not statistically significant compared to controls ($p < 0.05$).

Figure 2 shows that treatment with curcumin and its analogues resulted in marked reduction in nodule volumes. In the control group, DMH administration produced nodules with an average volume of 0.1522 mm^3. The administration of curcumin or its analogues resulted in ~ 98 % reduction in the average nodule volume ($p < 0.05$ for all treatment groups compared to controls). The nodule volume ranged from 0.0039 to 0.0051 mm^3 in the curcumin treatment group, 0.0033 to 0.0039 mm^3 in the GVT-0 treatment group, and 0.003 to 0.0037 mm^3 in the HGV-0 treatment group.

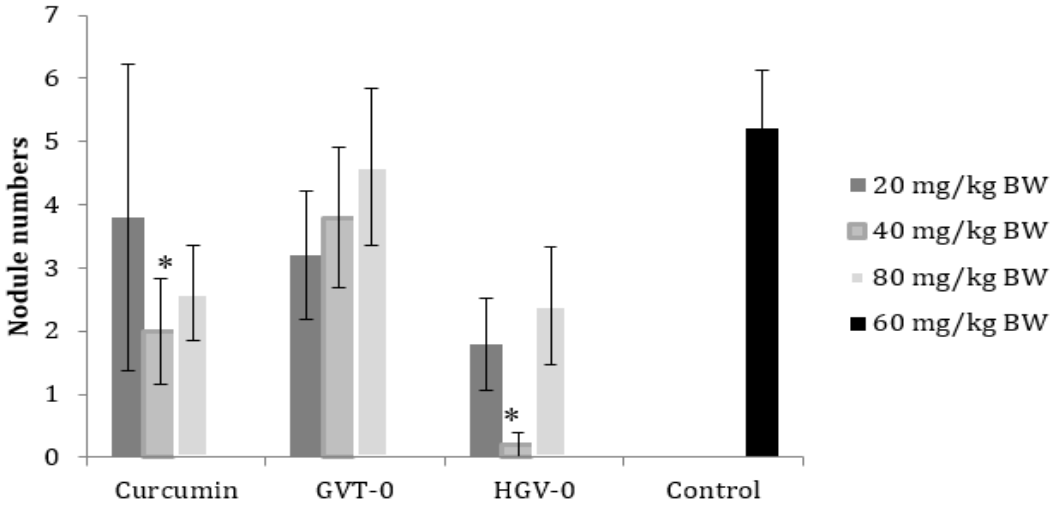

Figure 1: Effect of curcumin and its analogues on nodule numbers in DMH-induced colorectal tumors in rats after 15 weeks of treatments (n = 5). All data presented as mean ± SEM; *$p < 0.05$ compared to DMH control

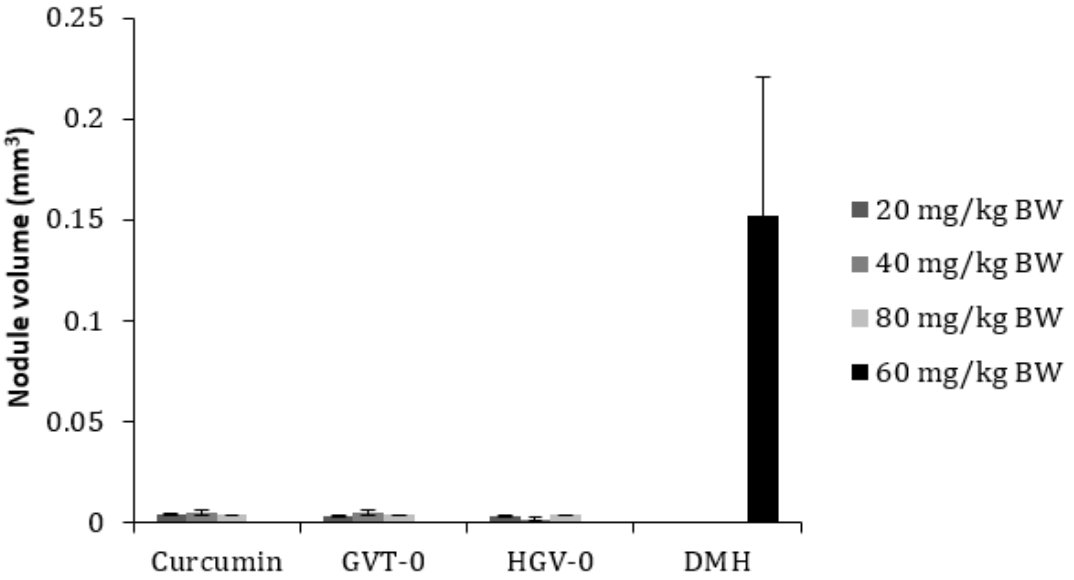

Figure 2: Effect of curcumin and its analogues on nodule volume in DMH-induced colorectal tumors in rats after 15 weeks of treatments (n = 5). All data are presented as mean ± SEM; *p < 0.05 compared to any other treatments

Expression of *adenomatous polyposis coli* (APC)

The effects of curcumin and its analogues on the expression of APC are shown in Table 1. APC expression was calculated in six visual fields using microscope with 400 × magnification. APC expression was observed via immunohistochemistry staining, observed as dark brown nuclei, in colorectal cancer. The results show that treatment with either 40 mg/kg curcumin, 40 mg/kg GVT-0, or 40 mg/kg HGV-0 for 15 weeks significantly inhibited the expression of APC compared to controls by 19.9, 15.6, and 29.8 %, respectively (p < 0.05).

Expression of COX-2

Treatment with curcumin, at doses of 20 and 40 mg/kg BW, as well as with HGV-0 at 20 and 40 mg/kg BW, significantly downregulated COX-2 expression. In contrast, GVT-0 did not significantly attenuate COX-2 expression (Table 2, Figure 3).

DISCUSSION

Previously, the curcumin analogues HGV-0 and GVT-0 have been shown to act as potent anti-inflammatory agents [12]. Local inflammation plays a role carcinogenesis. It is believed that COX-2 is among the inflammatory mediators that contribute the most in colorectal cancer pathogenesis [18,19]. Indeed, the level of COX-2 expression is associated with the number and volume of colorectal nodules [20].

In this present study, the chemopreventive effects of two curcumin analogues (HGV-0 and GVT-0) were compared to those of curcumin in a rat model of DMH-induced colorectal cancer.1,2-dimethylhydrazine (DMH) is an alkylating agent that is widely used to induce colorectal cancer in rodent models. DMH initiates and promotes colon carcinogenesis by inducing the formation of reactive carbonium ions, which then induce DNA methylation [21]. Three parameters were examined: (1) morphological changes (the presence and volume of nodules), (2) expression of mutant APC, and (3) expression of COX-2 in colorectal cancer.

The result of this study shows that curcumin, HGV-0, and GVT-0, at any given dose, significantly lowered the volume of nodules compared to controls. However, only HGV-0 at the dose of 40 mg/kg BW was effective to reduce the number of nodules as much as 96% compared to that in the control group. It is believed that HGV-0 chemoprevention in colorectal cancer is achieved through inhibition of expression of both mutated APC and COX-2. Cyclohexanone in the HGV-0 structure resembles a 6-carbon ring, that is formed by a β-diketone tautomer of curcumin, which is capable of inhibiting the production of prostaglandin E2 in DMH-induced colorectal cancer, and thus decreasing the amount of COX-2 protein. Reduced COX-2 protein not only results in downregulation of the inflammatory process, but also decreases the expression of APC gene mutations [19].

Table 1: Effect of GVT-0 and HGV-0 administration on the expression of APC mutants in rat colorectal tissue

Compound	Dose (mg/kg)	Immunoreactivity (IMR) (%)
Curcumin	20	76.60
	40	54.62
	80	67.58
Gamavutone-0	20	82.20
	40	57.52
	80	69.13
Hexagamavunone-0	20	77.71
	40	47.90
	80	63.41
DMH	60	68.20

All data are presented as mean ± SEM. * $p < 0.05$ compared to the DMH control group

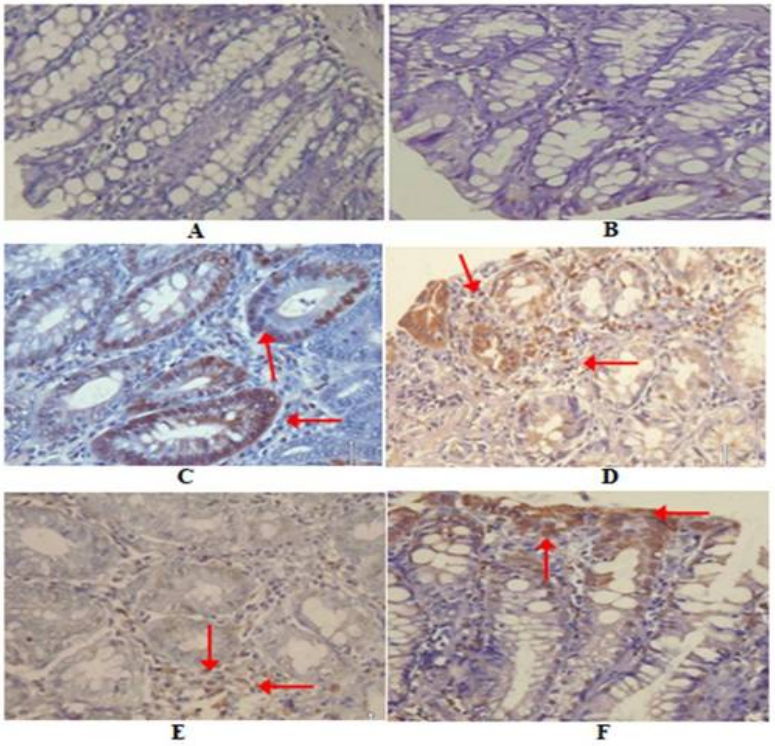

Figure 3: Microscopic images of rat colorectal tissue stained with APC rabbit polyclonal antibody with different treatments. A. Normal colorectal tissue; B. Normal colorectal tissue with antibody; C. Colorectal tissue with DMH treatment (60 mg/kg BW); D. DMH-treated colorectal tissue treated with curcumin (80 mg/kg BW); E. DMH-treated colorectal tissue treated with HGV-0 (40 mg/kg BW); F. DMH-treated colorectal tissue treated with GVT-0 (40 mg/kg BW). Magnification, 400 ×. Expression of mutant APC shown by red arrows

Table 2: Effect of GVT-0 and HGV-0 on COX-2 immunoreactivity in rat colorectal area

Compound	Dose (mg/kg BW)	Immunoreactivity(IMR) (%)
Curcumin	20	63.64
	40	62.50
	80	74.42
Gamavutone-0	20	68.75
	40	70.27
	80	75.86
Hexagamavunone-0	20	42.86
	40	40.00
	80	65.00
DMH	60	76.00

All data are presented as mean ± SEM. * $p < 0.05$ compared to the DMH control group.

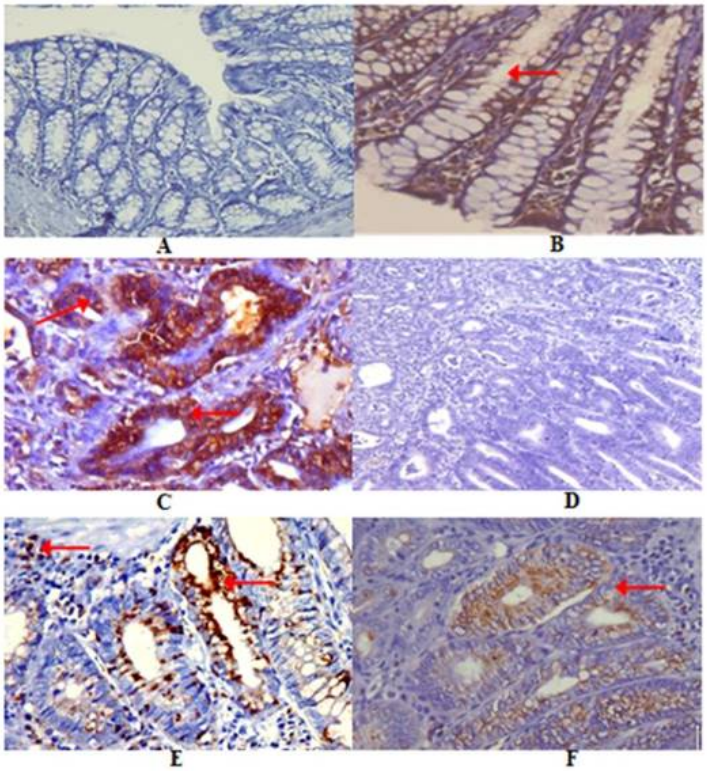

Figure 4: Microscopic rat colorectal with COX-2 rabbit polyclonal antibody staining with different treatment. A. Normal colorectal; B. Normal colorectal with antibody; C. DMH injection (60 mg/kg BW); D. DMH injection with curcumin (40 mg/kg BW) treatment; E. DMH injection with HGV-0 (40 mg/kg BW); F. DMH injection with GVT-0 (20 mg/kg BW). Magnification, 400 ×. COX-2 expression is indicated with a red arrow

Modification of the curcumin structure by removing one carbonyl group and adding a cyclohexanone molecule, which resembles the six-ring formed by the β-diketone curcumin tautomer, appears to improve the inhibitory activity of HGV-0 on cancer cell growth [22,23]. Indeed, the immunoreactivity of COX-2 expression was inhibited to a greater extent in colorectal rats treated with HGV-0 (40 mg/kg), than in those treated with curcumin and GVT-0.

Meanwhile, GVT-0 inhibited the enlargement of nodules (volume) without significant reduction in the number of nodules. This suggests that GVT-0 was ineffective in inhibiting the initiation of carcinogenesis but still has the capability to reduce the growth of nodules. This may be because GVT-0 lacks the ability to inhibit COX-2 upregulation; although, it can regulate the expression of mutated APC. The alteration of the β-diketone from curcumin into the monoketone-structure in GVT-0 is assumed to reduce COX-2 inhibition by this analogue, resulting in less chemopreventive activity in colorectal cancer [9]. This may indicate that COX-2 expression is required to initiate the generation of colorectal nodules, but the growth of nodules is more likely to depend on APC mutation. Further investigation is required to confirm this hypothesis.

CONCLUSION

Oral administration of the curcumin analogue HGV-0 (40 mg/kg) for 15 weeks significantly inhibited colorectal carcinogenesis, compared to curcumin and GVT-0 administration. This chemoprevention is thought to be mediated by HGV-0 action through the inhibition of mutated APC and COX-2 expression.

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by

the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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