Evaluation of Chemoprotective Effect of Quercetin from Argyreia speciosa against N-methyl-N-Nitro-N-Nitrosoguanidine and NaCl-Induced Gastric Carcinomas in Wistar Rats

Lubna Azmi1,2, Ila Shukla1, Shyam Sundar Gupta1, Aniruddh Chaudhary2, Padam Kant3, Narayan Prasad Yadav3, Chandana Venkateswara Rao1*

ABSTRACT

Objectives: This study was carried out to investigate the chemoprotective potential of Quercetin, an isolated compound from Argyreia speciosa, on N-methyl-N-nitro-N-nitrosoguanidine and NaCl-induced gastric carcinomas in Wistar rats. Methods: The rats were fed with a diet supplemented with 8% NaCl and simultaneously given N-methyl-N-nitro-N-nitrosoguanidine. After administration of the carcinogen, quercetin was administered. The whole stomach and a part of duodenum were sampled, cut open and tumors were recorded. The specimens were histopathologically investigated and the expression of surviving was examined with immunohistochemical analysis. Results and Conclusion: The treatment with quercetin significantly increases body weight in the rats after N-methyl-N-nitro-N-nitrosoguanidine administration. Survivin expression in glandular stomachs of normal rats, of rats in adenocarcinomas and quercetin at dose dependent manner treated rats were 0%, 90%, 75%, 33.3-25%, respectively. Compared with the survivin expression in negative rats, the differences were significant. Compared with the survivin expression in normal rats, the differences were significant. Histological observations of stomach tissues too correlated with the biochemical observations. These findings indicated that the Quercetin treatment could stimulate immunity activity in rats with N-methyl-N-nitro-N-nitrosoguanidine induced gastric carcinoma and have pronounced effect on survivin which is an attractive target for gastric cancer therapy. Key words: Argyreia speciosa, Gastric carcinoma, Immunochemistry, Quercetin.

INTRODUCTION

A wide variety of biological activities from medicinal plants have recently been reported, in addition to their traditional medicinal effects. Herbal medicines have attracted considerable interest as alternative cancer remedies because of their low toxicity and costs. Argyreia speciosa (Linn. f.), sweet is a popular Indian medicinal plant, which has long been used in traditional ayurvedic medicine. This plant is pharmacologically studied for nootropic, hepatoprotective, anti-inflammatory and wound healing activity. A wide range of phytochemical constituents have been isolated from this plant like quercetin, kaempferol and kaempferol 3-O-L-rhamnopyranoside, 7, 8, 3', 4', 5'-pentahydroxyflavone, 5-O-α-L-rhamnopyranoside and 7, 8, 3', 4', 5'-pentahydroxyflavone 5-O-β-D-glucopyranoside. Globally, gastric carcinoma is the 5th most common cancer with 952,000 cases diagnosed in 2012. It is more common in men and in developing countries. In India, the number of new stomach cancer cases in 2001 was estimated to be approximately 35,675 (n = 23,785 in men; 11,890 in women). Survivin, a member of the inhibitors of apoptosis protein (IAP) family, is a mitotic spindle-associated protein involved in linking mitotic spindle function to the activation of apoptosis in mammalian cells. The structure of full-length human survivin determined by X-ray crystallography is 2.7 Å. The structure forms a very unusual bow tie-shaped dimer. The unusual shape and dimensions of survivin suggest that it serves as an adaptor through its alpha-helical extensions. Just like other IAP members, survivin can suppress apoptosis through combination with Caspase3, Caspase7 by baculoviral IAP repeat (BIR). The common pathway of apoptosis is the activation of Caspase3, Caspase7 or Caspase6, hence high expression of survivin may protect cells from many apoptotic sig-
Toxicity studies and Dose determination

An acute oral toxicity study was performed according to the OECD guidelines for the testing of chemicals, Test No. 423 (OECD, 2001; acute oral toxicity-acute toxic class method). Rats (n=3) of either sex were selected by a random sampling technique for the acute toxicity study. Quercetin was administrated orally in increasing dose up to 2000 mg/kg. Pilot testing was carried out to determine the most effective dose of Quercetin (10, 50, 100, 200, 400 and 600 mg/kg) by damaging of stomach by ethanol.

N-methyl-N-nitro-N-nitrosoguanidine induced gastric Carcinoma

Fifty male 6-week-old wistar rats were divided into 2 groups (group α and group β). Forty rats in group α were fed with a diet supplemented with 8% NaCl for 20 weeks and simultaneously given N-methyl-N-nitro-N-nitrosoguanidine in drinking water at a concentration of 100 µg/mL for the first 17 weeks. After administration of the carcinogen, 100, 200 and 400 mg/kg of quercetin were administered orally once a day throughout the study to 30 rats of group α, subdivided (n=10 each) as α-100, α-200 and α-400 respectively. From week 18, these rats were given normal water. From week 21, these rats were fed with normal diet for 15 weeks. The other ten rats in group β were fed with normal diet for 35 weeks and served as the control. All the animals were killed at the end of week 35; the body weight of each rat was taken before sacrifice. The whole stomach and a part of duodenum were sampled and cut open along the greater curvature. The blood and spleen were collected by retro-orbital plexus followed by heart puncture and allowed to clot before centrifugation at 2500 g for 15 min at 40 °C and stored at -800 °C. Serum was used to analyze immunity activity and spleen was used to analyze proliferation rate. The number of tumors with their locations and sizes were recorded in detail. All the specimens were fixed in formalin and histopathologically investigated and the expression of surviving was examined with immunohistochemical analysis.

Immunohistochemical staining for survivin and assessment of its expression

Anti-survivin polyclonal antibody was purchased from Santa Cruz Company. Immunohistochemical analysis was carried out with the standard streptavidin-biotin-peroxidase (SP) complex technique using the Ultra-sensitive TM S-P kit (Maixin- Bio Company). Tumor cells served as positive control. Negative control slides were stained without primary antibody. To assess the expression of survivin in various samples examined, a 4-grade-method was established according to the mean percentage of positive tumor cells and their intensity. Moderately stained slides with a mean percentage of positive tumor cells no less than 30% were scored as positive (+). Moderately or intensively stained slides with a mean percentage of positive tumor cells more than 70% were scored as intensely positive (+++). Slightly or moderately stained slides with a mean percentage of positive tumor cells between 30%and 70% were scored as moderately positive (+). Slightly more moderately stained slides with a mean percentage of positive tumor cells less than 30% were scored as negative (-).

Biochemical analysis

Spleen Lymphoproliferation rate was measured according to the method of Girón-Pérez. Blood IgA, IgM and IgG levels were measured with a commercially available ELISA kit. Natural killer (NK) cells activity was measured according to the method of Li. Spleen CD4+/CD8+ was measured according to the method of Salem.
Table 1: Effect of Quercetin on Surviving expression of normal control and N-methyl-N-nitro-N-nitrosoguanidine induced gastric Carcinoma in rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Adenocarcinomas</th>
<th>Survivin expression</th>
<th>Positive rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group β Normal</td>
<td>β(n=10 )</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Group α Negative</td>
<td>α(n=10)</td>
<td>10</td>
<td>90%</td>
</tr>
<tr>
<td>Quercetin Treatment</td>
<td>α-100 (n=10)</td>
<td>8</td>
<td>75%</td>
</tr>
<tr>
<td></td>
<td>α-200 (n=10)</td>
<td>6</td>
<td>33.3%</td>
</tr>
<tr>
<td></td>
<td>α-400 (n=10)</td>
<td>4</td>
<td>25%</td>
</tr>
</tbody>
</table>

Statistical analysis
Software Stata (version 6.0, STATA Corp, College Station) was applied to compare the rates. One-way ANOVA followed by Duncan’s multiple range tests was used to compare the parameters among the different groups.

RESULTS
The isolated quercetin from ethanolic leaf extract of *A. speciosa* was found to be a yellowish green mixture having melting point 307-317 °C. Its spectral data showed UV (MeOH) absorbance at λmax 255, 372 nm; IR (KBr) νmax 3439, 3100, 2370, 1615, 1610, 1504, 1457, 1364, 1295, 1260, 1182, 1110, 803 cm⁻¹; 1 H NMR (CDCl₃, 300 MHz) δ 5.352 (1H, brs, H-6), 3.653 (1H, dd, 5.4, 8.5 Hz, H-3a) 1.262 (3H, brs, Me-21) 1.243 (3H, brs, Me-19), 1.031 (3H, brs, Me-29), 0.924 (3H, d, J=6.1 Hz, Me-21), 0.861 (3H, d, J=6.30 Hz, Me-26) 0.843 (3H, d, J=6.3Hz, Me-27), 0.821 (3H, brs, Me-30), 0.662 (3H, brs, Me-18); 13C (CDCl₃, 300 MHz) δ 130.5 (Et-C2), 119.3 (Et-C3), 185.0 (carbonyl C4), 156.6 (ArC5), 95.6 (Ar-C6), 162.2 (Ar-C7), 92.3 (Ar-C8), 151.3 (Ar-C9), 101.0 (Ar-C10), 127.5 (Ar-C1'), 113.6 (Ar-2'), 143.4 (Ar-3'), 142.6(Ar-C4'), 116.1 (Ar-5), 118.5 (Ar-6') and -ve ion DART MS m/z (rel. int.) 301.07 [M]- (C15H10O7) (100), 270 (10), 152 (6), 134 (12), 112 (20), 97 (82), 82 (32), 63 (42).

By the end of week 35, neoplastic foci were found in antral mucosa in total 10 rats of group α which were histologically determined to be adenocarcinomas. Of these 10 rats, the total number of adenocarcinomas was 10, and 9 were very well differentiated, which could be classified as intestinal type according to Lauren classification. 90% positive staining was in cytoplasm of tumor cells. Whereas group α-100, α-200 and α-400 shows 75%, 33.3% and 25% positive rate respectively for survivin was in cytoplasm of tumor cells. Whereas group α-100, α-200 and α-400 shows 75%, 33.3% and 25% positive rate respectively for survivin was in cytoplasm of tumor cells.

DISCUSSION
Survivin is expressed in a series of human cancers, and it has been widely accepted that survivin is highly related to the onset and development of cancer. Some scholars concluded that survivin could only be found in tumor, but others thought it could be found in precancerous lesions. Our results supported the latter. Survivin is not only expressed in cancer tissues but also in damaged tissues. The expression of survivin occurs before the formation of adenocarcinomas, and is an early event in carcinoma development. Recently, some studies have partly explained why survivin was highly expressed in cancer. According to the study of, the anti-apoptotic gene, survivin, is a p53- repressed gene. Chromatin immunoprecipitations indicate that wild type p53 binds survivin promoter (Figure 2). The quercetin administration dose-dependently significantly (P<0.01) increased proliferation of spleen lymphocytes, dose-dependently. We also found that quercetin administration dose-dependently significantly (P<0.01) increased blood IL-2 levels and NK activities (Figure 2).

There was significant (P<0.01) decrease in blood IgA, IgG and IgM levels of quercetin treated control rats (α-100, α-200, α-400 respectively) as compared with the negative control rats. The quercetin treatment dose-dependently significantly (P<0.01) increased the blood IgA, IgG and IgM levels of rats with stomach cancer (Figure 3).

There was significant (P<0.01) decrease in CD4+ and CD4+/CD8+ of negative control rats as compared with the normal control rats (group β) (Figure 4). However, there was not significant (P>0.01) change in CD8+ between all groups. quercetin treatment resulted in significant (P<0.01) increase in CD4+ and CD4+/CD8+ when compared to negative control rats (Figure 4).

Figure 1: Effect of Quercetin on body weight of normal control and N-methyl-N-nitro-N-nitrosoguanidine induced gastric Carcinoma in rats. Values are expressed as mean ± SEM of 10 rats in each group. *P<0.01, compared with respective normal control group. ++P<0.01 compared with negative control group.

Figure 2: The quercetin administration dose-dependently significantly (P<0.01) increased proliferation of spleen lymphocytes, dose-dependently.
survivin expression and apoptosis inhibition. Abnormal apoptosis leads to carcinogenesis. In this study, we adopted experimental gastric carcinoma models to discuss the effect of quercetin on survivin expression of gastric cancer. Survivin was expressed in negative control group rats, the positive rate rose to 90%. These data suggest that high expression of survivin is a common phenomenon in gastric cancer and inhibition of apoptosis resulted from survivin expression may play an important role in carcinogenesis. Survivin expression in glandular stomachs of normal rats, of rats in adenocarcinomas and quercetin at dose dependent manner treated rats were 0%, 90%, 75%, 33.3-25%, respectively. Compared with the survivin expression in negative rats, the differences were significant. In our animal experiment, 95.7% of the induced gastric carcinomas were intestinal type, and we did find atrophy and dysplasia lesions during the induction period. These data suggest that our rat model could simulate the development of human gastric carcinoma (intestinal type). These data indicate a rising trend of survivin expression during the development of gastric cancer; play an important role of survivin expression in tumor formation which was briefly explained in case of negative control group as cancer cells were developed of survivin expression and tumor formation was increased. Survivin expression and tumor formation decreased as concentration of quercetin was increased in dose dependent manner. According to our preclinical data, age and prognostic factors such as different tumor size, tumor depth, and lymph node metastasis or disease stage have no significant correlation with survivin expression, which indicates that survivin has little impact on tumor biological behaviour.

**Figure 2:** Effect of Quercetin on (A) spleen lymphocytes proliferation, (B) IL-2 and (C) NK activity of normal control and N-methyl-N-nitro-N-nitrosoguanidine induced gastric Carcinoma in rats. Values are expressed as mean ± SEM of 10 rats in each group. *P<0.01 compared with respective normal control group. ++P<0.01 compared with negative control group.

**Figure 3:** Effect of Quercetin on blood IgA, IgM and IgG levels of normal control and N-methyl-N-nitro-N-nitrosoguanidine induced gastric Carcinoma in rats. Values are expressed as mean ± SEM of 10 rats in each group. *P<0.01 compared with respective normal control group. ++P<0.01 compared with negative control group.

**Figure 4:** Effect of Quercetin on (A) CD4+, (B) CD8+ and (C) CD4+/CD8+ activity of normal control and N-methyl-N-nitro-N-nitrosoguanidine induced gastric Carcinoma in rats. Values are expressed as mean ± SEM of 10 rats in each group. *P<0.01 compared with respective normal control group. ++P<0.01 compared with negative control group.
Survivin plays an important role in gastric carcinoma and is a key molecule of cell cycle, mitosis and apoptosis. Moreover, inhibition of survivin function will result in cell apoptosis. The antisense oligonucleotide targeting survivin expression sensitizes lung cancer cells to chemotherapy. In vitro experiments showed that flavonoids can directly activate leukocytes, stimulating their phagocytic, cytotoxic, and anti-microbial activity. In addition, they can stimulate the production of proinflammatory mediators, such as cytokines and chemokines. Our work showed that quercetin obtained from A. speciosa explains the mechanism of immune modulating activity.

CONCLUSION

Together, these results indicated that the Quercetin treatment could stimulate immunity activity in rats with N-methyl-N-nitro-N-nitrosoguanidine induced gastric carcinoma and have pronounced effect on survivin which is an attractive target for gastric cancer therapy.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS USED


REFERENCES

cordandfrom=exportandto=exporthand=LM36992328%5CnCht. http://findit.library.jhu.edu/resolv
er?sid=EMBASE&findissn=10079327andti=andtitl=Expression+of+survivin+
in+human+gastric+carcinoma+and+gastric+carcinoma+model+of+rats
dstti.
fas.2007.07.002.
91:11<2026::AI:D;1-CVRICR12328>3.0.CO;2-E.
Introdction: Present study was done to explore the chemo-protective potential of quercetin, an isolated compound from *Argyreia speciosa*, on N-methyl-N-Nitro-N-Nitrosoguanidine and NaCl-induced gastric carcinomas in Wistar rats. Methods: After administration of the carcinogen, quercetin was administered. The specimens were histopathologically investigated and the expression of survivin was examined with immuno-histochemical analysis. Results: The treatment with quercetin significantly increases body weight in the rats after N-methyl-N-nitro-N-nitrosoguanidine administration. Compared with the survivin expression in negative rats and normal rats, in both the case differences were significant. Histological observations of stomach tissues too correlated with the biochemical observations. Conclusions: These findings indicated that the Quercetin treatment could stimulate immunity activity in rats with N-methyl-N-nitro-N-nitrosoguanidine induced gastric carcinoma and have pronounced effect on survivin which is an attractive target for gastric cancer therapy.