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Original Article

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 Azmi[1,2], Ila Shukla[1], Shyam Sundar Gupta[1], Aniruddh Chaudhary[2], Padam Kant[2], Narayan Prasad Yadav[3], Chandana Venkateswara Rao[1*]

ABSTRACT

Objectives: This study was carried out to investigate 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:** 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.

Lubna Azmi [1,2], Ila Shukla[1], Shyam Sundar Gupta[1], Aniruddh Chaudhary[2], Padam Kant[2], Narayan Prasad Yadav[3], Chandana Venkateswara Rao[1*]

[1]Pharmacognosy and Ethnopharmacology Division, CSIR-National Botanical Research Institute, Lucknow, Uttar Pradesh, INDIA.
[2]Department of Chemistry, University of Lucknow, Lucknow, INDIA.
[3]Botany and Pharmacognosy Department, CSIR-Central Institute of Medicinal and Aromatic Plants, P.O. CIMAP, Lucknow, INDIA.

Correspondence

Dr. Ch V Rao

(Principle Scientist) CSIR-National Botanical Research Institute, Rana Pratap Marg, Lucknow, ZIP Code: 226001, INDIA.

Phone No: + 91-9450659295

E mail Id: chvrao72@yahoo.com

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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,[1] immunomodulatory,[2]hepatoprotective,[3]antioxidant,anti-inflammatory[4] and wound healing activity.[5] A wide range of phytochemical constituents have been isolated from this plant like quercetin, kaempferol and kaempferol 3-O-L-rhamnopyranoside,[6] 7, 8, 3',4',5'-pentahydroxyflavone, 5-O-α-L-rhamnopyranoside and 7, 8, 3',4',5'-pentahydroxyflavone 5-O-β-D-glucopyranoside,[7]

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,[8] The structure of full-length human survivin determined by X-ray crystallography is 2.7 A°,[9] 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,[10] Just like other IAP members, survivin can suppress apoptosis through combination with Caspase3, Caspase7 by baculoviral IAP repeat (BIR).[11,12] 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-

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nals and help cells survive.[13,14] Now, substantial data have shown that inhibition of apoptosis plays a great role in carcinogenesis,[15] so survivin may be an important factor in the development of cancer.[9] It has reported that survivin is undetectable in terminally differentiated adult tissues and becomes prominently expressed in transformed cell lines and in most common human cancers of lung, colon, pancreas, prostate and breast.[16] Some data indicate that high expression of survivin is correlated with poor prognosis and chemotherapy resistance.[17]

A. speciosa, which is unique in having high yield of secondary metabolites. It is phytochemically characterized by particular abundance of flavonoids (i.e. quercetin) specimens of which are kept in the herbarium of CSIR-CIMAP, Lucknow, India) display various biological properties.[7] In gastrointestinal area they act as secretoinhibitory, cytoprotective and antioxidant agents.[4] In this study, we investigated the effect of chemo protective potential of quercetin isolated from *A. speciosa* on expression of survivin in gastric carcinoma models of rats.

MATERIALS AND METHODS

Collection and identification of plant materials

A. speciosa was collected by the authors from botanical garden of National Botanical research institute, India. Fully developed leaf samples, sixth to seventh from the top of nearly 150-day-old plants were collected during the first week of March 2015. It was identified by taxonomist, and the specimen was deposited in the Herbarium of CSIR-National Botanical Research Institute, Lucknow, India.

Extract preparation and Standardization

The fresh leaves were air-dried and pulverized into coarse powder using a hammer mill. The powdered plant material (500 g) was extracted with petroleum ether three times to remove fatty materials for 24 h with immediate shaking. This was followed by extraction with 65% of ethanol by cold percolation at room temperature for 48 h. The extract was separated by filtration and concentrated under vaccum using rotary evaporator (Buchi USA) at 40°C to yield 9.65% (w/w) of ethanol leaf extract of *A. speciosa* (ELEAS). Preliminary qualitative photochemical screening of *A. speciosa* has given the positive test for alkaloids, glycosides, flavonoids, terpenoids, saponins and steroids.

Isolation of Quercetin

Quercetin was isolated from ethanolic leaf extract of *A. speciosa*. The crude product was partitioned in distilled water and diethyl ether. The aqueous part was treated with 20% of $NaHCO_3$ (pH 8-9) and partitioned with chloroform. The pH of aqueous part was changed to 3-4 by treatment with 6N HCL and partitioned with ethyl acetate. Fractions were applied in the form of band using Linomat IV applicator for HPTLC analysis for isolation of quercetin which was identified by IR, NMR and mass spectroscopy.

Experimental Animals

Albino rats (175.40±4.42 g) were obtained from the CSIR-CDRI, Lucknow, India. They were kept individually in stainless steel cages at temperature of (22±2) °C, relative humidity 50-60% with a 12 h light/ dark cycle respectively for one week before and during experiment and access standard rodent pellet diet. Food was withdrawn 1 8-24 h before the experiment through water was allowed *ad libitum* and allocated to different experimental groups All studies were performed according to the guidelines of the Institutional Animal Ethics Committee, CPCSEA, India (Reg. No. 1732/GO/ Re/S/13/CPCSEA).

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 to separate serum 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.[18]

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 (-).[18]

Biochemical analysis

Spleen Lymphoproliferation rate was measured according to the method of Girón-Pérez.[19] 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.[20] Spleen CD4+/CD8+ was measured according to the method of Salem.[21]

Table 1: Effect of Quercetin on Surviving expression of normal control and N-methyl-N-nitro-N-nitrosoguanidine induced gastric Carcinoma in rats.

Treatment			Adenocarcinomas	Survivin expression	Positive rate %
Group β	Normal control	β(n=10)	0	0	0%
Group α	Negative control	α(n=10)	10	9	90%
	Quercetin	α-100 (n=10)	8	6	75%
	Treatment control	α-200 (n=10)	6	2	33.3%
		α-400 (n=10)	4	1	25%

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 λ_{max} 255, 372 nm; IR (KBr) υ_{max} 3429, 3100, 2370, 1651, 1610, 1504, 1457, 1364, 1295, 1260, 1182, 1110, 803 cm^{-1} ; 1 H NMR ($CDCl_3$, 300 MHz) δ 5.352 (1H, brs, H-6), 3.653 (1H,dd, 5.4,8.5 Hz, H-3α) 1.262 (3H, brs, Me-28), 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 ($CDCl_3$, 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 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 reported less surviving expression as concentration of quercetin was increased upto dose 400 mg/kg. No survivin expression was detected in antral mucosa of normal rats (group β). As shown in Table 1. At week 35, all rats in both group α and β were killed and investigated. In case of adenocarcinomas tissue adjacent to tumor shows effected. Tissues adjacent to tumor were defined as the tissues 5 mm away from the edge of tumor a $P<0.05$ *vs* control group, b $P<0.001$ *vs* control group, c $P<0.01$ *vs.* control group.

Figure: 1 shows that body weight (initial and final) and stomach weights of group β and α rat. The final body weight of normal control group β showed (245.2±7.6) g which was significantly decreased to (176.0±8.3) g ($p<0.001$) comparatively in negative control group. In Quercetin treated groups α-100 α-200, α-400 rats the final body weights become significantly increased to 214.0±9.6 ($p<0.01$), 239.3±7.2 ($p<0.001$) and 242.1±8 ($p<0.001$) respectively.

To well understanding how Quercetin conditionally promotes antibody responses *in vivo*, proliferation studies on purified populations of spleen lymphocytes have been performed. In this study, we found that proliferation of antigen receptor-stimulated rat peripheral blood lymphocyte cells was significantly ($P<0.01$) inhibited in rats with stomach cancer (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).

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.[8] Some scholars concluded that survivin could only be found in tumor,[22] but others thought it could be found in precancerous lesions.[23] 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.[24] the anti-apoptotic gene, survivin, is a p53- repressed gene. Chromatin immunoprecipitations indicate that wild type p53 binds survivin promoter

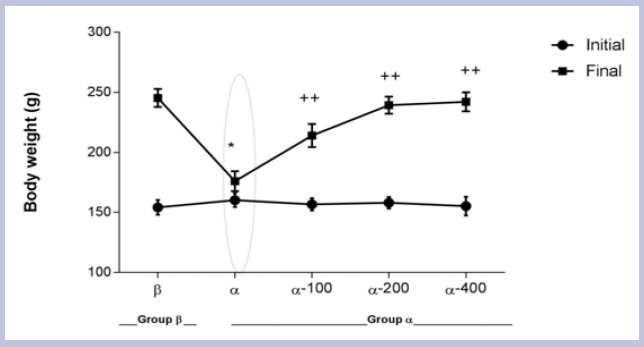

Figure 1: Effect of Quercetin on body weight of normal control and N-methyl-N-nitro-N-nitrosoguanidine induced gastric Carcinoma in rats. Values are expressed as mean ± SEM of 10 rats in each group. *P<0.01, compared with respective normal control group. ++P<0.01 compared with negative control group.

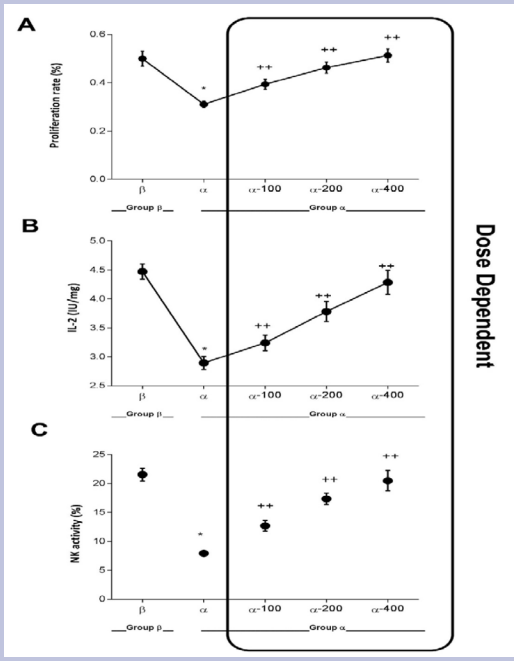

Figure 2: Effect of Quercetin on (A) spleen lymphocytes proliferation, (B) IL-2 and (C) NK activity of normal control and N-methyl-N-nitro-N-nitrosoguanidine induced gastric Carcinoma in rats. Values are expressed as mean ± SEM of 10 rats in each group. * P<0.01 compared with respective normal control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

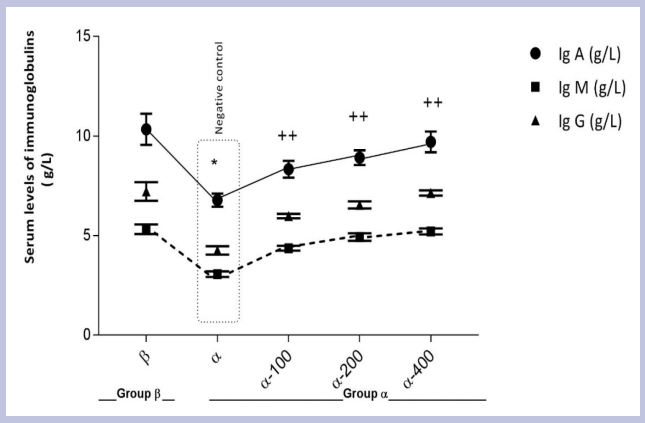

Figure 3: Effect of Quercetin on blood IgA, IgM and IgG levels of normal control and N-methyl-N-nitro-N-nitrosoguanidine induced gastric Carcinoma in rats. Values are expressed as mean ± SEM of 10 rats in each group. *P<0.01 compared with respective normal control group. ++P<0.01 compared with negative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

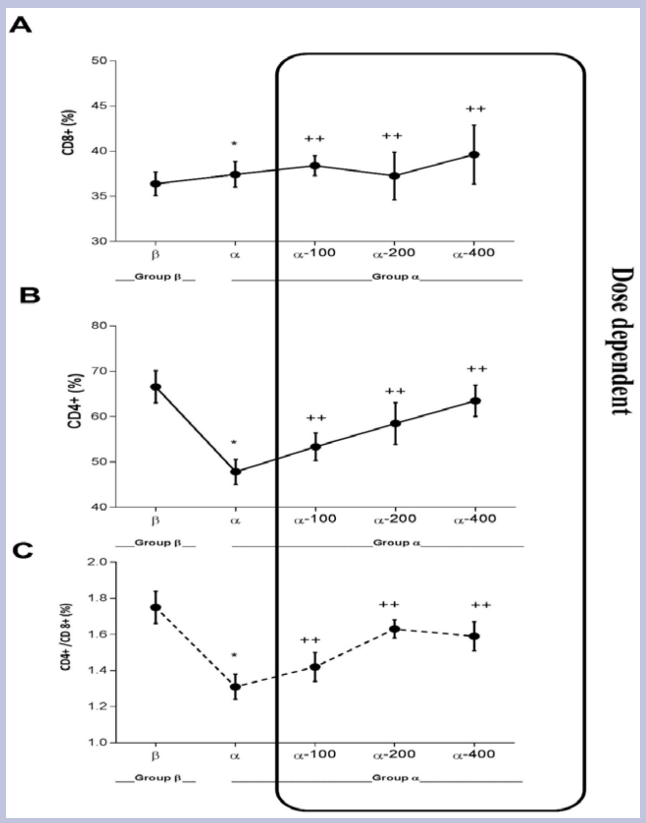

Figure 4: Effect of Quercetin on (A) CD4+, (B) CD8+ and (C) CD4+/CD8+ activity of normal control and N-methyl-N-nitro-N-nitrosoguanidine induced gastric Carcinoma in rats. Values are expressed as mean ± SEM of 10 rats in each group. *P<0.01 compared with respective normal control group. ++P<0.01 compared with negative control group.

in vivo, which results in transcriptional repression.[25] study implicated that wild-type p53 suppresses survivin expression at both mRNA and protein levels. It is widely accepted that mutated p53 loses its function as a tumor inhibitor and this may contribute to the loss of inhibition to surviving.[13] Due to the high incidence of p53 mutation in gastric cancer, we put forward the hypothesis that long term effect of carcinogen should lead to p53 or other important gene damage, which results in survivin expression and apoptosis inhibition. Abnormal apoptosis leads to carcinogenesis.[9] In this study, we adopted experimental gastric carcinoma models to discuss the effect of quercetinon 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).[18] 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.

Survivin plays an important role in gastric carcinoma and is a key molecule of cell cycle, mitosis and apoptosis.[26] Moreover, inhibition of survivin function will result in cell apoptosis. The antisense oligonucleotide targeting surviving expression sensitizes lung cancer cells to chemotherapy.[27] *In vitro* experiments showed that flavonoids can directly activate leukocytes, stimulating their phagocytic, cytotoxic, and antimicrobial 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

HPTLC: High-Performance Thin-Layer Chromatography; **IR:** Infra Red; **NMR:** Nuclear Magnetic Resonance; **CPCSEA:** Committee for the Purpose of Control and Supervision of Experiments on Animals; **ELISA:** Enzyme-Linked Immunosorbent Assay; **OECD:** Organization for Economic Co-Operation and Development; **ANOVA:** Analysis of Variance.

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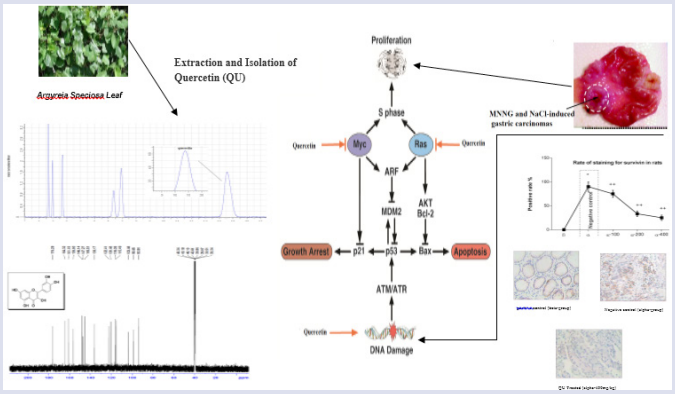
GRAPHICAL ABSTRACT



SUMMARY

- Introduction: 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.

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