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Evaluation of Immunomodulatory activity of the flavanoid from *Kigelia africana*

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ABSTRACT

Modulation of the immune responses to alleviate the diseases has been of interest for many years. Thus a real need exists to protect our immune systems and lead healthier lives. Hence the present study is aimed to evaluate the immunomodulatory activity of Flavanoid of *Kigelia africana.* The effect of flavanoid of *Kigelia africana* on the immune system of rats and mice was evaluated by using different experimental models such asmice lethality test, Serum immunoglobulin level, Haemagglutination reaction, hypersensitivity reaction, and delayed type hypersensitivity reaction test. Flavanoid of *Kigelia africana* was administered orally at low dose and high dose of 100mg/kg/day, poand 200 mg/kg/day, po respectively and Levamisole (2.5 mg/kg/day, po) was used as standard drug. Flavanoid of *Kigelia africana*in both doses increased the levels of serum immunoglobulin and prevented the mortality induced by bovine *Pasteurella multocida* in mice. Exhibits a dose related increase in the early hypersensitivity reaction and Delayed type hypersensitivity reaction to the SRBC antigen. It also resulted in a significant increase in the antibody titer value, to SRBC, in experimental animals. Hence, it was concluded that flavanoid of *Kigelia africana* increases both humoral immunity and cell mediated immunity.

Introduction

Herbal drugs are known to possess immunomodulatory properties and generally act by stimulating both specific and nonspecific immunity. Many plants used in traditional medicine have immunomodulating activities. Natural adjuvants, synthetic agents, antibody reagents are used as immunosuppressive and immunostimulative agents. But there are major limitation to the general use of these agents such as increased risk of infection and generalized effect throughout the immune system. Immunosuppression is a major drawback in conventional therapy of cancer such as radiation and chemotherapy. Both these method have sever side effect such as nausea, vomiting, alopecia, mucosal ulceration etc. Modulation of immune responses to alleviate the diseases has been of interest for many years and the concept of 'Rasayana' in Ayurveda is based on related principles. Immunostimulation in a drug-induced immunosuppression model and immunosuppression in an experimental hyperreactivity model by the same preparation can be said to be true immunomodulation. Apart from being specifically stimulatory or suppressive, certain agents have been shown to possess activity to normalize or modulate pathophysiological processes and are hence called immunomodulatory agents. The concept of immunomodulation relates to nonspecific activation of the function and efficiency of macrophages, granulocytes, complement, natural killer cells and lymphocytes and also to the production ofvarious effector molecules generated by activated cells. It is expected that theses nonspecific effects giveprotection against different pathogens including bacteria, viruses, fungi etc. and constitute an alternative to conventional chemotherapy.[1, 2]

Due to wide range of effector mechanism possessed by various groups of immune cells and its ability to exert effects with exquisite specificity, immune system provide a good target in cancer therapy. Involvement of the host immune system in the

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control of cancer progression has been suspected but remained inconclusive for many years. Innate immunity, which according to the immune surveillance theory is responsible for early detection and elimination of malignant cells, may be inefficient patients who develop malignancy. Evidence is convincing that individuals who are older, who have been on immuno-suppressive medications over prolonged periods of time, or have underlying immune abnormalities, such as an autoimmune disease or a chronic infection(e.g., AIDS) are particularly at risk of malignancy.

Kigelia africana (*Lam*) Benth. (Bignoniaceae) is widespread across India and Africa and is found in most wet savannah and riverline areas. Growing over 20 m high, it is semi-deciduous with grey-brown smooth bark. The fruits are large grey-green "sausages" about 30- 60 cm long which hang on stalks from the tree. The fresh fruit is poisonous and strongly purgative; for safety reasons, fruits are best prepared for consumption by drying, roasting and fermentation. Scientific literature confirms the validity of many of these traditional uses due to the presence of numerous secondary metabolites. These compounds include iridoids, flavonoids, fatty acids, sterols, glycosides and naphthoquinones.[3]

The present study was undertaken to evaluate the effect of flavanoid of *Kigelia africana* on the immune system using different experimental models to substantiate the traditional claim. The study helps in understanding the effect of flavanoid of *Kigelia africana* on different components on the immune system.

Materials and methods

Experimental animals

Albino Wistar rats weighing between (200-250 gm.) and Swiss albino mice weighing between (25-35 gm.) was used. Institutional Animal Ethics Committee approved the experimental protocol; animals were maintained under standard conditions in an animal house approved by Committee for the Purpose of Control, and Supervision on Experiments on Animals (CPCSEA). The animals were given pellet food (Lipton India Ltd., Mumbai, India) and water *ad libitum.* (Reg.no.1564/PO/a/11/CPCSEA)

Plant material

The leaves of *Kigelia africana* were collected from Thirupathi forest region Thirupathi District, Andhra Pradesh, INDIA in the month of June 2013. This plant species were authenticated by Dr. K. Madhava Chetty, Assistant professor, Department of Botany, Sri Venkateswara University, Thirupathi, Andhra Pradesh, India, where a voucher specimen has been preserved for future identification.

Preparation of Extract

100gm powdered leaves parts were subjected to successive extraction in a Soxhlet extractor using methyl alcohol. The extract obtained was concentrated in a rotary shaker and evaporated to dryness to get constant weight.[4]

Isolation of flavanoid

The method employed for the isolation of flavonoids was of Jang *et al* (2003) with modifications. 200gm of *Kigelia africana* powder were defatted with hexane is a Soxhlet extractor. The marc was pressed and dried. The defatted powder was extracted with methanolin a Soxhlet extractor for 16 hours. The methanol extract was filtered and concentrated to dryness in a rotary shaker evaporator. The concentrated extract was dissolved in 80% methanol with stirring and filtered. Methanol fractions were subjected to chemical analysis. Methanol extract fraction was concentrated kept in refrigerator overnight. As there was no crystal formed, the solvent was evaporated to dryness. The residue answered the test for flavonoids. [5, 6]

Chemicals

Leishmann's stain, Indian ink and glutaraldehyde were purchased from Merck (Mumbai, India). WBC diluting fluid, zinc sulphate and barium chloride were from Nice Chemicals (Cochin, India). Cyclophosphamide (Endoxan Injection) was from German Remedies (Mumbai, India).*Pasteurella multocida* of bovine origin and its vaccine were obtained from Institute of Animal Health and Veterinary Biologicals (Bangalore, India).

Antigen

Fresh blood was collected from sheep sacrificed in the local slaughter house. Sheep red blood cells (SRBCs) were washed three times in large volumes of Alsever's solution and adjusted to a concentration of 0.5×10^9 cells/ml for immunization and challenge.[7]

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Selection of Dose and Treatment period

The animals were divided into four groups consisting of six animals each. The first group served as control(vehicle 1 ml/100 g, *po*), the second group received Levamisole at a dose of (2.5mg/Kg, *po*).Third and fourth group received the low dose (100mg/Kg, *po*) and high dose of flavanoid of *Kigelia africana* respectively.

Acute toxicity studies

The acute toxicity study was carried out according to the limit test described in the OPPTS guidelines. Briefly, atest dose of 2 g/kg and 5 g/kg were given orally to themice. The extract was found to be safe at the dose of 2 g/kg, po. Hence, 1/10th and 1/20th of the safe dose corresponding to 100 mg/kg and

200mg/kg orally were selected as low and high dose respectively.[7, 8]

Delayed type hypersensitivity (DTH) response

Six animals per group were immunized on day 0 by (i.p).Administration of 0.5 x 10^9 SRBC/rat and challenged by a subcutaneous administration of 0.025 x 10^9SRBC/ml into right hind foot pad on day +14. The flavanoid of *Kigelia africana* was administered orally from day -14 until day +13. DTH response was measured at 24 h after SRBC challenge on day +14 and expressed as mean percent increase in paw volume (plethysmometrically).[9]

Hypersensitivity reaction

Hypersensitivity reaction which measures cellular immunity
Hypersensitivity reaction to SRBC was induced in rats, following the prescribed method. The Flavanoid of *Kigelia africana*(in doses of 100 and 200mg/kg, body weight) was administered to the animals (test group) orally for five days and the vehicle was administered to the control animals. Each group consisted of six rats – three male and three female. The Flavanoid of *Kigelia africana*was administered orally on each of the two days prior to the immunization, on the day of the immunization and on each of the two days after the immunization (i.e., Days –2, -1, 0, +1. +2). The rats were immunized by injecting 0.1 ml of SRBC subcutaneously into the right hind footpad on day 0. The animals were challenged seven days later by injecting the same amount of SRBC into the left hind footpad. The thickness of the left hind footpad was measured with a micrometer at 4 h and 24 h after the challenge.[10,11]

Mice lethality test

Swiss albino mice were treated with different extracts orvehicle orally for 21 days. On the 7th and 17th day of thetreatment, the animals were immunized with haemorrhagic septic aemic vaccine (HS vaccine). On the21st day, the animals were challenged subcutaneouslywith 0.2 ml of lethal dose (25 x LD50) of *Pasteurellamultocida* (bovine origin) containing 107cells per ml. Theanimals were observed for a period of 72 hr and the Mortality ratio was determined using the formula: Mortality ratio: Number of animals dead/ Total number of animals.

Effect on serum Immunoglobulin

The drugs were administered to female albino rats orally for 21 days. Six hours after the last dose of drug, blood was collected and the serum was used for estimation of immunoglobulin levels using method devised by Mullen(1975).Briefly, for each serum sample to be analyzed, a control tube containing 6 ml of distilled water and a test tube containing 6 ml of zinc sulphate solution were prepared. To each, 0.1 ml of serum was added from a pipette. [12]
They were inverted to enable complete mixing of the reagents and left to stand for 1 hr at room temperature. The first tube served as blank and the second tube was taken as sample. The turbidity developed was measured using a digital nepheloturbidity meter. The turbidity obtained (sample-blank) was compared with that obtained with standard barium sulphate ($BaSO_4$) solution. The standard $BaSO_4$ solution was prepared by adding 3 ml of barium chloride solution (1.15% w/v) to 97 ml of 0.2 N sulphuric acids. The turbidity obtained with this solution was expressed as 20 zinc sulphate turbidity (ZST) units.

Haemagglutination reaction

Haemagglutination reaction which measures the humoral immunity. The flavanoid of *Kigelia africana*(in doses of 100 and 200 mg/kg, body weight) was administered to the animals (test group) orally for five days and the vehicle was administered to the control animals. Each group consisted of six rats – three male and three female. The Flavanoid of *Kigelia africana* was administered orally on each of the two days prior to the immunization, on the day of the immunization and on each of the two days after the immunization (i.e., Days –2, -1, 0, +1. +2). The rats were immunized by injecting 0.5 ml of SRBCs intraperitoneally (ip) on the day of the immunization. Blood samples were collected by retro-orbital puncture on the tenth day after the immunization. Antibody levels were determined by the Haemagglutination technique. The antibody titer was determined by a two-fold serial dilution of one volume (100 µL) of serum and one volume (100 µL) of 0.1% bovine serum albumin (BSA) in saline. One volume (100 µL) of 0.1 % SRBCs in BSA in saline was added and the tubes were mixed thoroughly. They were allowed to settle at room temperature for about 60-90 min until the control tube showed a negative pattern (a small button formation). The value of the highest serum dilution showing visible Haemagglutination was taken as the antibody titer.[13]

Statistical analysis

The results are expressed as mean ±S.E.M. Data analysed by one way ANOVA followed by the "Turkey's Multiple Comparison Test". P values are $P<0.05^{*}$, $P<0.01^{**}$, $P<0.001^{***}$ as compared with control were considered

Results

Acute oral toxicity

The LD_{50} was found to be 2000mg/kg. So 1/10th and 1/20th of dose was taken for the study.

Preliminary phytochemical screening

The presence of various phytoconstituents of the extracts was detected by phytochemical screening. The KA found to contain

Steroids and triterpenes, phenolic compounds, tannins, and flavonoids.

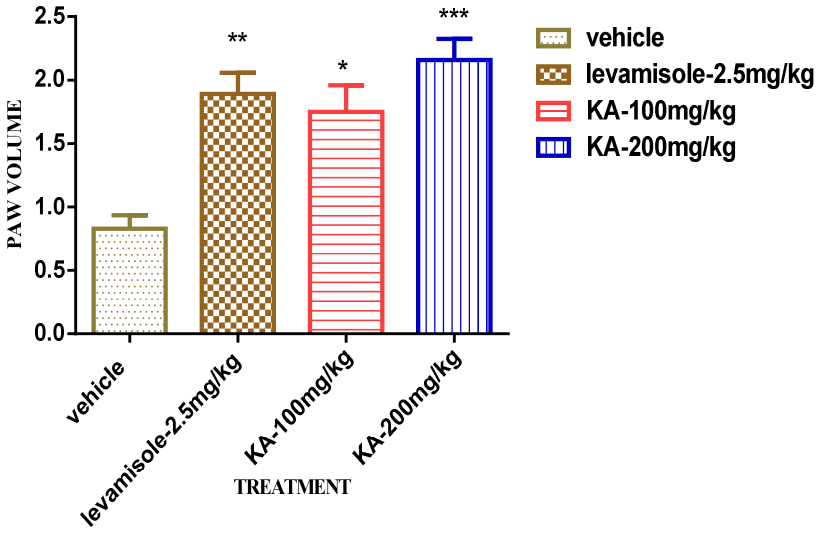
Delayed type hypersensitivity (DTH) response

In Delayed type hypersensitivity reaction test (DTH), the DTH response directly correlates with cell-mediated immunity (CMI). Flavanoid of *Kigelia africana*at both the doses and Levamisole showed(p<0.05*, P<0.001***,P<0.01***) significantly increased Delayed Type Hypersensitivity Reaction when compared to control group.

Table 1:Effect of Flavanoid of KA on Delayed Type Hypersensitivity Reaction in Wister rats

Group	Treatment	Delayed type hypersensitivity reaction-24 hours(paw volume)
I	Vehicle (1 ml /Kg,po/day/28days)	0.83 ± 0.10
II	LMS (2.5 mg/Kg,po/day/28days)	1.89 ± 0.27**
III	FKA (100 mg /Kg,po/day/28days)	1.75 ± 0.21*
IV	FKA (200 mg /Kg,po/day/28days)	2.16 ± 0.17***

Values are expressed as mean ± SEM. N=6, $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001 when compared to control. Statistically analysed by one way ANOVA followed by Tukey-Kramer multiple comparison test.



Values are expressed as mean ± SEM. N=6, $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001 when compared to control.

Fig no 1: Effect of Flavanoid of KA on Delayed Type Hypersensitivity Reaction in Wister rats

Hypersensitivity reaction

In the early hypersensitivity reaction, the antigen antibody formed immune complexes, which are known to induce local inflammation with increased vascular permeability, edema and

infiltration of PMN leucocytes.Flavanoid of *Kigelia africana* at both the doses and Levamisole showed significant ($^{***}P$<0.001) increase in early hypersensitivity reaction to SRBC.

Table 2: Effect of Flavanoid of KA on Hypersensitivity Reaction in Wister rats

Group	Treatment	Paw thickness in mm (4 hours)	Paw thickness in mm (24 hours)
I	Vehicle (1 ml /Kg,po/day/5days)	0.411 ± 0.012	0.081 ± 0.03
II	LMS (2.5 mg/Kg,po/day/5days)	0.662 ± 0.021	0.371 ± 0.04***
III	FKA (100 mg /Kg,po/day/5days)	0.781 ± 0.051	0.509 ± 0.02***
IV	FKA (200 mg /Kg,po/day/5days)	0.922 ± 0.028	0.763 ± 0.03***

Values are expressed as mean ± SEM. n=6. $^{***}P$<0.001 when compared to control.
Statistically analysed by one way ANOVA followed by Tukey-Kramer multiple comparison test.

Mice lethality test

The mouse lethality testis one of the widely used tests to evaluate serological responses in animals immunized with vaccines.Flavanoid of *Kigelia africana*at 100mg/Kg, po/day/28 days showed 50% and 200mg/Kg, po/day/28 days showed 33.33% reduction in the mortality ratio when compared to control signifying enhancement in antibody production.

Effect on serum immunoglobulin

Both the doses of *Flavanoid of Kigelia africana* and LMS showed a significant ($^{**}P$<0.01, $^{***}P$<0.001) increase in the serum immunoglobulin levels when compared to control.

Table 3: Effect of Flavanoid of KA on Mice lethality test inSwiss albino mice

Treatment	Day-1 No of dead animals	Day-2 No of dead animals	Day-3 No of dead animals	MORTALITY RATIO
Distilled water + Pasteurellamultocida(0.2ml/single dose)	3	2	-	100%
LMS(2.5mg/kg)+ Haemorrhagic septicaemic vaccine(0.1 ml) + Pasteurellamultocida(0.2ml/single dose)	-	1	1	33.33%
FKA(100mg/kg)+ Haemorrhagic septicaemic vaccine(0.1 ml) + Pasteurellamultocida(0.2ml/single dose)	-	1	2	50%
FKA (200mg/kg)+ Haemorrhagic septicaemic vaccine(0.1 ml) + Pasteurellamultocida (0.2ml/single dose)	-	1	1	33.3%
Haemorrhagic septicaemic vaccine(0.1 ml) + Pasteurellamultocida(0.2ml/single dose)	-	3	2	83.33%

Table 4: Effect of flavanoid of *Kigelia africana* on serum immunoglobulin levels in Wistar rats

Treatment	Serum immunoglobulin level(ZST-UNITS)
Control-Distilled Water (1 ml /Kg,*po*/day/21days)	21.12 ± 0.55
LMS (2.5 mg/Kg,*po*/day/21days)	38.14 ± 1.40***
FKA (100 mg /Kg,*po*/day/21days)	28.4 ± 0.85**
FKA (200 mg /Kg,*po*/day/21days)	33.04 ± 1.33***

Values are expressed as mean ± SEM. n=6, $^{**}P<0.01$, $^{***}P<0.001$ when compared to control.
Statistically analysed by one way AOVA followed by Tukey-Kramer multiple comparison test.

Haemagglutination reaction

Antibody molecules which are secreted by plasma cells mediate the humoral immune response.Flavanoid of *Kigelia africana* at both the doses and Levamisole showed a significantly ($P<0.01$,$P<0.05$) increase in the Haemagglutination titer value signifying increase in antibody titre.

Table 5: Effect of flavanoid of *Kigelia africana* on Haemagglutination titer level in Wistar rats

Treatment	HA titer value (µL/100 µL)
Vehicle (1 ml /Kg,po/day/5days)	5.854 ± 3.27
LMS (2.5 mg/Kg,po/day/5days)	10.14 ± 1.62*
FKA (100 mg /Kg,po/day/5days)	13.94 ± 1.84 **
FKA (200 mg /Kg,po/day/5days)	16.08 ± 1.96 **

Values are expressed as mean ± SEM. n=6 $^{*}p<0.05$ $^{**}P<0.01$ when compared to control.
Statistically analysed by one way AOVA followed by Tukey-Kramer multiple comparison test.

Discussion

The results of the present study suggest that Flavanoid of *Kigelia africana* potentiates humoral immunity as shown by its effect in serum immunoglobulin levels, mice lethality test and Haemagglutination reaction and it also potentiates cell mediated immunity as shown by its effect on Hypersensitivity reaction and delayed Hypersensitivity reaction.

The estimation of serum immunoglobulin levels was used to evaluate the increase in serum immunoglobulin production after the administration of the drugs. Immunoglobulin are antibodies that react specifically with the antigen. The zinc sulphate turbidity test is used to gain a rough estimation of the amount of Immunoglobulin present in the serum. Zinc sulphate causes precipitation of the Immunoglobulin making the solution cloudy.

A lack of cloudiness signifies lack of immunoglobulins.[14, 15] The turbidity is expressed as ZST units, which in turn indicate the amount of immunoglobulin present in the sample. Flavanoid of *Kigelia africana* at both the doses (100 & 200mg/kg) showed a significant increase in the serum immunoglobulin levels.

The mouse lethality testis one of the widely used tests to evaluate serological responses in animals immunized with vaccines. *Pasteurella multocida* is pathogenic to mice. The mouse lethality test involves injecting mice with the vaccine prior to the administration of the bacterial culture and determining the mortality ratio.[16]The vaccination will cause production of antibodies. If the drug has an ability to enhance the production of antibodies to such an extent that antibodies produced can counter the pathogen, then the animals survive. Flavanoid of *Kigelia africana* at 100mg/Kg, po/day/28 days showed 50% and 200mg/Kg, *po*/day/28 days showed 33.33% reduction in the mortality ratio when compared to control signifying enhancement in antibody production.[17, 18]

Antibody molecules which are secreted by plasma cells mediate the humoral immune response. This augmentation of the humoral response to SRBC indicated an enhanced responsiveness of the macrophages and T and B lymphocyte subsets involved in antibody synthesis. Flavanoid of *Kigelia africana* at both the doses and Levamisole showed a significantly increase in the Haemagglutination titer value signifying increase in antibody titre.[19, 20] In the early hypersensitivity reaction, the antigen antibody formed immune complexes, which are known to induce local inflammation with increased vascular permeability, edema and infiltration of PMN leucocytes. The early increase in vascular permeability as well as neutrophils influx has been ascribed to the complement C_{5a} fragment which is activated by this immune complex.[21]Flavanoid of *Kigelia africana* at both the doses and Levamisole showed that increased early hypersensitivity reaction to SRBC. This indicated the stimulatory effect of FKA on chemotaxis dependent leucocytes migration.[22, 23] In Delayed type hypersensitivity reaction test (DTH), the DTH response directly correlates with cell-mediated immunity (CMI). The mechanism behind this elevated DTH during the CMI responses could be due to sensitized T-lymphocytes. When challenged by the antigen, they are converted to lymphoblast and secrete a variety of molecules including proinflammatory lymphokines, attracting more scavenger cells to the site of reaction. Flavanoid of *Kigelia africana*at both the doses and Levamisole showed significantly increased Delayed Type Hypersensitivity Reactionwhen compared to control group.[24-26] Findings of the present study showed an overall stimulatory effect of Flavanoid of *Kigelia africana*on both humoral and cellular immunity.

Conclusion

In the present study, flavanoid of *Kigelia africana* showed immunostimulant activity. Thus, it can be concluded that Flavanoid of *Kigelia africana* has therapeutic potential and could be served as an effective immunomodulatory candidate without any side effects and support the traditional claim of *Kigelia africana* for medicinal purposes.

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Conflict of interest statement

We declare that we have no conflict of interest.

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