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Original article

Pharmacokinetics and antiangiogenic studies of potassium koetjapate in rats

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ABSTRACT

Purpose: Koetjapic acid is an active compound of a traditional medicinal plant, *Sandoricum koetjape*. Although koetjapic acid has a promising anticancer potential, yet it is highly insoluble in aqueous solutions. To increase aqueous solubility of koetjapic acid, we have previously reported a chemical modification of koetjapic acid to potassium koetjapate (KKA). However, pharmacokinetics of KKA has not been studied. In this study, pharmacokinetics and antiangiogenic efficacy of KKA are investigated.

Methods: Pharmacokinetics of KKA was studied after intravenous and oral administration in SD rats using HPLC. Anti-angiogenic efficacy of KKA was investigated in rat aorta, human endothelial cells (EA.hy926) and nude mice implanted with matrigel.

Results: Pharmacokinetic study revealed that KKA was readily absorbed into blood and stayed for a long time in the body with T_{max} 2.89 ± 0.12 h, C_{max} 7.24 ± 0.36 μg/mL and $T_{1/2}$ 1.46 ± 0.03 h. The pharmacological results showed that KKA significantly suppressed sprouting of microvessels in rat aorta with IC_{50} 18.4 ± 4.2 μM and demonstrated remarkable inhibition of major endothelial functions such as migration, differentiation and VEGF expression in endothelial cells. Further, KKA significantly inhibited vascularization in matrigel plugs implanted in nude mice.

Conclusions: The results indicate that bioabsorption of KKA from oral route was considerably efficient with longer retention in body than compared to that of the intravenous route. Further, improved antiangiogenic activity of KKA was recorded which could probably be due to its increased solubility and bioavailability. The results revealed that KKA inhibits angiogenesis by suppressing endothelial functions and expression of VEGF.

1. Introduction

It is a well-known fact that poor solubility and dissolution in the gastrointestinal fluids are limiting factors for many drugs which pose a critical problem in bioavailability upon oral administration. Solubility is one of the vital factors to achieve desired concentration of drug in systemic circulation and to produce an effective pharmacological response. Low aqueous solubility is a major hurdle which is usually encountered during development of pharmaceutical formulations. In order for a drug to be absorbed, it has to be converted into a solution form at the site of absorption. Therefore, solubility has become the biggest challenge for pharmaceutical researchers working in the drug delivery system.

Sandoricum koetjape Merr. (Meliaceae) is a traditional medicinal plant and native to Malaysia, Cambodia and Southern Laos. Previously, we have reported that koetjapic acid (KA) is the active principle of *S. koetjape* which contributes towards the anticancer activity of this herb against human colon cancer cell line [1]. However, it was observed that KA has poor solubility in water as well as in other cell culture permitted solvents *i.e.*, dimethyl sulfoxide, thus posing an obstacle to further research and its clinical application. In order to enhance solubility of KA, we have reported a chemical modification of KA into its salt form,

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potassium koetjapate [2]. Potassium koetjapate (KKA) showed enhanced aqueous solubility and thereby it exhibited *in vitro* efficacy.

In the present study bioavailability of potassium koetjapate (KKA) was investigated in Sprague Dawley Rats using HPLC. In addition, KKA was tested for its antiangiogenic activity using a series of ex vivo and *in vitro* angiogenesis based experimental models such as rat aortic ring assay, and the proliferation, differentiation and migration properties of human endothelial cells.

2. Material and methods

2.1. Animals

Male Sprague Dawley rats weighing 350 − 410 g were obtained from the Animal Research and Service Centre (ARASC), Universiti Sains Malaysia, Penang, Malaysia. The animals were maintained in a 12 h light-dark cycle, at room temperature and were allowed free access to standard food pellets. Animal ethics Reference number: USM/IACUC/2018/(111)(913).

2.2. Preparation of different concentrations of potassium koetjapate (KKA)

A stock solution of KKA was prepared by dissolving 10 mg of KKA in 1 mL distilled water to obtain 10 mg/mL concentration. For cell *in vitro* studies, further dilutions were prepared using culture media following the serial dilution method.

2.3. Evaluation of bioavailability of potassium koetjapate (KKA)

The study was conducted according to a two-way crossover study design. Healthy and adult SD rats were fasted overnight with free access to water prior to the experimentation. Food was only allowed 4 h after the sampling of blood. In the first occasion, 3 rats (group I) were randomized to receive 50 mg/kg of KKA (dissolved in water) administered intravenously *via* the tail vein, while three other rats (group II) received orally 50 mg/kg of the same sample. After a washout period of 2 weeks, the animals from group (I) were given 50 mg/kg of KKA orally, whilst those from group (II) received 50 mg/kg intravenously of KKA. The rats were placed in animal restraining cages during blood collection and blood samples of 0.25–3 mL were withdrawn from the tail vein at 0, 5, 15, 30, 60, 90,120, 150, 180, 240, 300, 720, and 1440 min after intravenous injection or oral administration of the test sample. The blood samples were collected into microcentrifuge tubes containing 20 µL of heparin solution. All blood samples were centrifuged at 3000 rpm for 10 min. The resulting plasma samples were kept at −20 °C prior to HPLC analysis. An aliquot of 200 µL plasma was added in a 1.5 mL micro-centrifuge tube, and mixed with 200 µL HPLC grade methanol containing 10 µg/mL of betulinic acid (BA) as an internal standard. The mixture was vortexed for 2 min followed by centrifugation at 8000 rpm for 10 min. The supernatant was transferred into a 0.15 mL glass conical insert. For analysis, 20 µL of supernatant was injected into the HPLC system. Chromatographic condition for HPLC analysis of KA and BA was set according to the method described previously [3]. The chromatographic analysis was carried out on a ZORBAX Eclipse Plus C8 column (250 × 4.6 mm internal diameter ×5 µm particles size) (Agilent, USA) at 30 °C and the injection sample (10 µL) was eluted with an isocratic mobile phase comprising of acetonitrile-water (9:1) pH 2.5 (with trifluoroacetic acid). Flow rate was adjusted to 1 mL/min and detection was carried out at 210 nm wavelength.

2.3.1. Assay validation

KKA calibration curve was constructed by spiking KKA-free pooled rat plasma (200 µL) with a known amount of KKA at a concentration range of 0.3125 − 10 µg/mL. Followed by the addition of 200 µL methanol containing 10 µg/mL BA for extraction of KKA from spiked pooled plasma. These plasma standards were also used for the determination of Lower Limit of Detection (LLOD) and Lower Limit of Quantification (LLOQ), the extraction recovery, intra-day and inter-day precision and accuracy (n = 6) of the method [4].

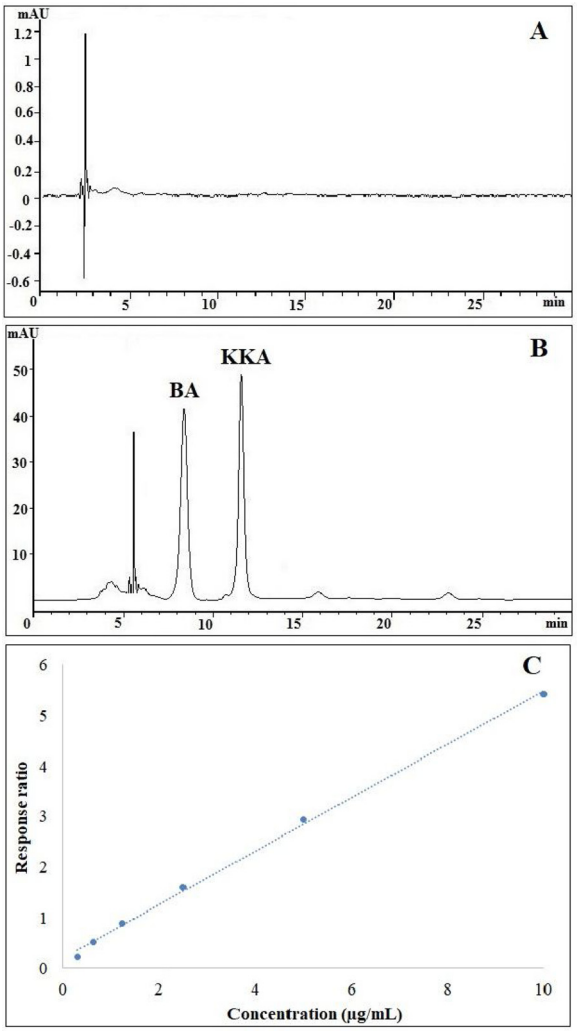


Fig. 1. HPLC Method Development.
A) HPLC chromatogram of blank rat plasma.
B) HPLC chromatogram of rat plasma spiked with betulinic acid (BA) and potassium koetjapate (KKA) at retention times (R_t) values 8.3 and 11.7 min, respectively.
Standard calibration curve of potassium koetjapate (KKA) in rat plasma depicting the linear regression curve.

2.3.2. Determination of pharmacokinetic parameters

The pharmacokinetic parameters estimated from the data of IV administration were, area under plasma concentration-time curve ($AUC_{0\rightarrow\infty}$), elimination rate constant (Ke), biological half-life ($t_{1/2}$), volume of distribution (Vd), and clearance (CL). The value of $AUC_{0\rightarrow\infty}$ (µg h/mL) was determined by adding the area from time zero to the last sampling time ($AUC_{0\rightarrow t}$) and to the area from last sampling time to infinity ($AUC_{t\rightarrow\infty}$). The Ke (h^{-1}) was calculated using the relationship, $t_{1/2} = \ln 2/Ke$ while V_d (L/kg) was calculated from the relationship, $V_d = dose/Ke \times AUC_{0\rightarrow\infty}$. Clearance CL (L/kg h) was calculated from the relationship, $CL = dose/AUC_{0\rightarrow\infty}$. Whereas from the data of oral administration, area under plasma concentration-time curve $AUC_{0\rightarrow24}$ (µg h/mL), maximum concentration (C_{max}) and time to reach T_{max} to maximum concentration were calculated. The percentage of absolute bioavailability of the standard compounds was estimated from the following equation [5]:

Table 1
Calibration data, LOD and LOQ of the reported HPLC method.

Compound	LOD (μg/mL)	LOQ (μg/mL)	Linearity range (μg/mL)	Equation	R2 Value
KA	0.10	0.31	0.3125 − 10	y = 0.5298x + 0.1972	0.999

Table 2
Plasma extraction recovery of koetjapic acid (KA).

Name	Concentration	Recovery	
	(μg/mL)	Mean	RSD (%)
KA	0.3125	96.41	0.87
	0.625	96.58	1.34
	1.25	97.97	1.56
	2.5	99.34	2.10
	5	95.60	1.64
	10	98.82	1.39

Table 3
Intra-day and inter-day precision (n = 6).

Compounds	Concentration	Within Day		Between day	
	(μg/mL)	Accuracy (% of true value)	RSD (%)	Accuracy (% of true value)	RSD (%)
KA	0.3125	102.70	0.45	100.36	1.65
	0.625	96.79	1.87	97.75	1.31
	1.25	92.33	1.07	91.69	1.59
	2.5	96.45	1.79	95.06	1.08
	5	97.95	0.89	97.42	0.96
	10	101.98	2.00	101.35	1.80

%Absolute bioavailability (F) = (AUC for oral/AUC for IV) × (IV dose/oral dose) × 100

The samples were analyzed in triplicates and results were presented as mean ± SEM, n = 6.

2.4. Anti-angiogenic studies

2.4.1. Culture conditions and maintenance of cell lines

Human umbilical vein (EA.hy926, ATCC® CRL-2922) cell lines were purchased from the American Type Culture Collection (Rockville, MD) and were cultured and maintained in Roswell Park Memorial Institute-1640 (RPMI-1640). Media was appended with 5–10 % heat inactivated fetal bovine serum (Kansas, USA) and 1% penicillin/streptomycin [2]. Additionally, EA.hy926 cells were propagated in endothelial cell medium supplemented with 1 % endothelial cell growth supplements (ECGS). All cell lines were cultured in a humidified incubator at 37 °C supplied by 5 % CO_2, and were monitored frequently to detect the presence of any contamination such as bacterial or fungal growth. Hemocytometer was used to count the cells as described by the earlier method [6].

2.4.2. Assessment of proliferation of endothelial cells

MTT assay was used to study the effect of KKA on proliferation of human endothelial cells (EA.hy926) [7]. The assay plates were read using a microtiter plate reader (Multiskan Ascent microplate reader, Thermolab system 354, Finland) at 570 nm absorbance. Culture media alone was used as a negative control. KA alone was also used as a reference for comparison.

2.4.3. Cell migration assay

The assay was carried out as described previously [8]. Briefly, EA.hy926 cells were plated in 6 well plates until a confluent monolayer was formed after which a wound was created with 200 μL micropipette tips. The detached cells were removed by washing them twice with PBS (MediaTech, USA) and the plates were treated with KKA. The wounds were photographed after 12 and 18 h. The width of the cell-free wound was measured under an inverted light microscope supplied with Leica Quin computerized imaging system. Ten fields per well were photographed and a minimum of 30 readings per field were measured.

The percentage of wound closure was then calculated relative to zero time using the formula: % wound closure = 1 − (the width at the indicated times (h) /the width at zero time 100 %:

The results were presented as average ± SD, (n = 3).

2.4.4. Tube formation assay

The ability of EA.hy926 to form tube-like structures was investigated on Matrigel matrix (BD Bioscience, USA) [9]. Briefly, the matrigel matrix was allowed to polymerise for 45 min at 37 °C and 5% CO_2. Then EA.hy926 were trypsinized and seeded (3×10^4 cells per

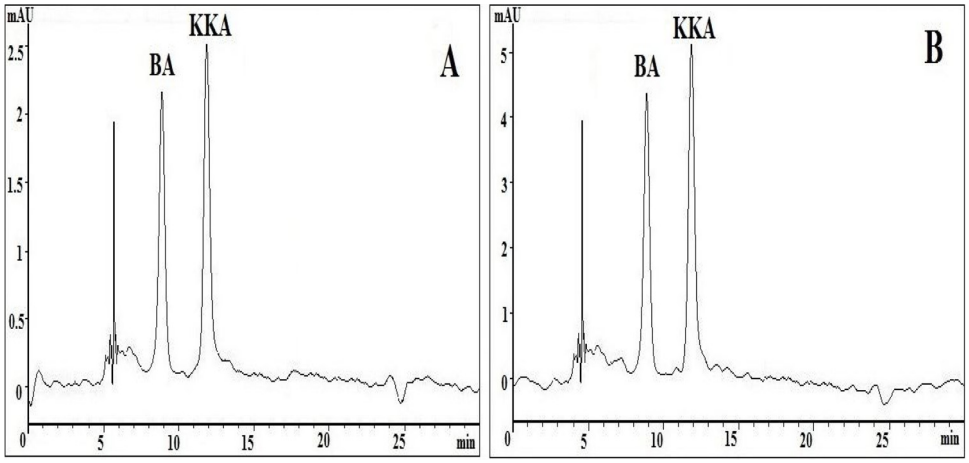


Fig. 2. HPLC profile obtained in pharmacokinetic study.
A) Chromatogram of KKA (50 mg/kg body weight) in rat plasma at 2 h after oral administration.
B) Chromatogram of KKA (50 mg/kg body weight) in rat plasma at 2 h after intravenous administration.

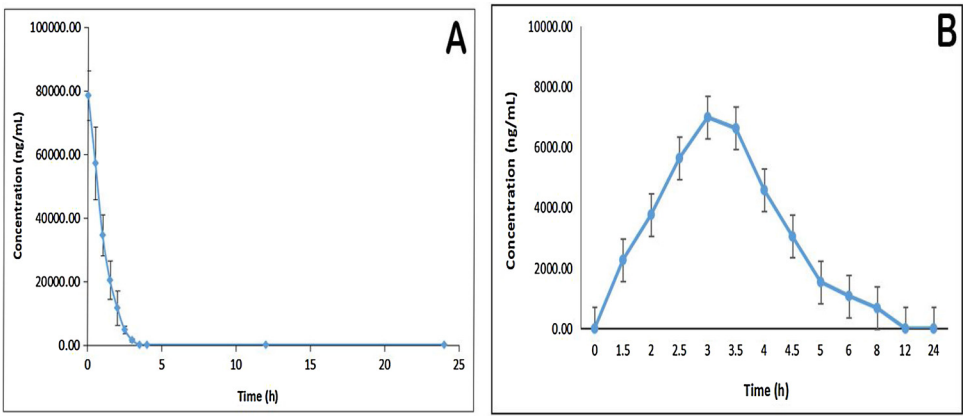


Fig. 3. The pharmacokinetic profiles of KKA (50 mg/kg) in rats.
A) Mean plasma concentration of KKA vs. time profile after intravenous administration at in rat plasma (data presented as mean ± S.E.M, n = 6).
B) Mean plasma concentration of KKA vs. time profile after oral administration at in rat plasma (data presented as mean ± S.E.M, n = 6).

Table 4
Pharmacokinetic parameters of koetjapic acid (KA) in rat plasma after intravenous administration (n = 6).

Data	Unit	KA
$AUC_{(24-\infty)}$	μg h/mL	84.87 ± 4.71
Ke	h^{-1}	1.30 ± 0.04
$t_{1/2}$	h	0.54 ± 0.02
V_d	L/kg h	0.15 ± 0.01
CL	L/kg h	0.20 ± 0.01

Table 5
Pharmacokinetic parameters of koetjapic acid (KA) in rat plasma after oral administration) (n = 6).

Data	Unit	koetjapic acid
C_{max}	μg/mL	7.24 ± 0.36
T_{max}	h	2.67 ± 0.12
Oral bioavailability	%	32.14 ± 3.06

well) in 100 μL of RPMI-1640 (GE Healthcare HyClone, USA) culture medium with triplicates of different concentrations of KKA. After 6 h, the tube-like structures were visualized and imaged under light microscopy at 4× magnification. The area occupied by the capillary-like structures was then measured on treatment and control groups by Image J software and the results were shown as a quantified of width and length of tube-like structure of treated and untreated cells The results were demonstrated as a average ± SD, (n = 3).

2.5. Ex vivo rat aortic ring assay

Rat Aortic Ring Assay was conducted with the following method [10]. In short, aortic rings with 1 mm width taken from thoracic aortas of 12–14 weeks old male Sprague Dawley rats were seeded separately in a 48-well plate in 300 μL serum free M199 media mixed with 3 mg/mL fibrinogen and 5 mg/mL aprotinin. 50 NIH U ml^{-1}thrombin in 0.15 M NaCl were pipetted in each well. Afterwards incubated for 90 min at 37 °C, followed by adding 6 different concentrations of KKA dissolved in 0.3 mL M 199 medium containing 20 % HIFBS, 0.1 % ε-aminocaproic acid, 1% L-Glutamine, 2.5 μg/mL amphotericin B, and 60 μg/mL gentamicin (Sigma, Germany), to each well while botulinic acid and DS water were used as positive and negative controls, respectively. The medium was changed with a fresh one mixed with the compound on the fourth day. On the next day, aortic rings were imaged using an inverted light microscope at 4× magnification. Then, the length of blood vessels outgrowth from the primary tissue explants was measured using the Leica Quin software package following the methods described by Nicosia [7,11]. The results were illustrated as mean percent inhibition to the negative control ± SD, (n = 3). The inhibition of blood vessels formation was calculated according to the following formula:

Blood vessels inhibition = (1- (A0/A)) * 100

In which, A0 = distance of blood vessels growth in treated rings with KKA in μm, A = distance of blood vessels growth in the control in μm.

2.6. Assessment of VEGF levels in endothelial cells

The effect of KKA on the release of angiogenic cytokine (VEGF) from endothelial cells was determined using a human VEGF-165 ELISA kit (Raybio, USA) as per the manufacturer's guidelines. In short, 60–70 % confluent cultures of EA.hy926 were treated with 75 and 150 μM of KKA and 75 μM and 150 μM of KA. Then, cell lysates were prepared using the supplied cell lysis buffer. This assay employs an antibody specific for human VEGF coated on a 96-well plate. Standards and EA.hy926 lysates were pipetted into the plate. VEGF present in the samples bounded with the immobilized antibody. The wells were washed and a biotinylated anti-human VEGF antibody was added before the wells were washed again. Subsequently, HRP-conjugated streptavidin was added to the wells. After washing, the coloring agent Tetramethylbenzidine substrate solution was pipetted to the wells. The Stop Solution (H_2SO_4) was added to each well, and the intensity of the color was measured at 450 nm. At the time of experiment a calibration curve of VEGF standard was prepared. The concentration of VEGF in cell lysates was calculated using the log-log regression equation of the best fit line of the standard calibration curve; (y = 0.0099 × 0.6137, R2 = 0.996).

2.7. In vivo matrigel plug assay

In vivo antiangiogenic activity of KKA was studied using the matrigel sponge model of angiogenesis followed by the protocol [12]. In brief, NU/Nu immunocompromised nude mice were injected subcutaneously with 0.3 mL of Matrigel appended with 100 μL of HCT-116 cells, (1×10^6 / ml RPMI-1640), close to the abdominal midline. Next, the mice were treated orally in three different doses: 50, 100, and 200 mg/kg of KKA daily for two weeks. Distilled water and imatinib (100 mg/kg) were used as the negative and positive controls, respectively. In the last day of the experiment, the animals were euthanized and Matrigel plugs were excised. The Matrigel plugs were fixed in paraffin and cut. Then, paraffin block sections (5 μm at thickness) were stained with hematoxylin and eosin (H&E). Subsequently, the number of blood vessels in all sections was measured. Ten microscopic fields per slide were studied at 20× and 40× magnification and photomicrographic images were taken by a digital camera. Then, the numbers of blood vessels were compared between the treatment and control groups [13].

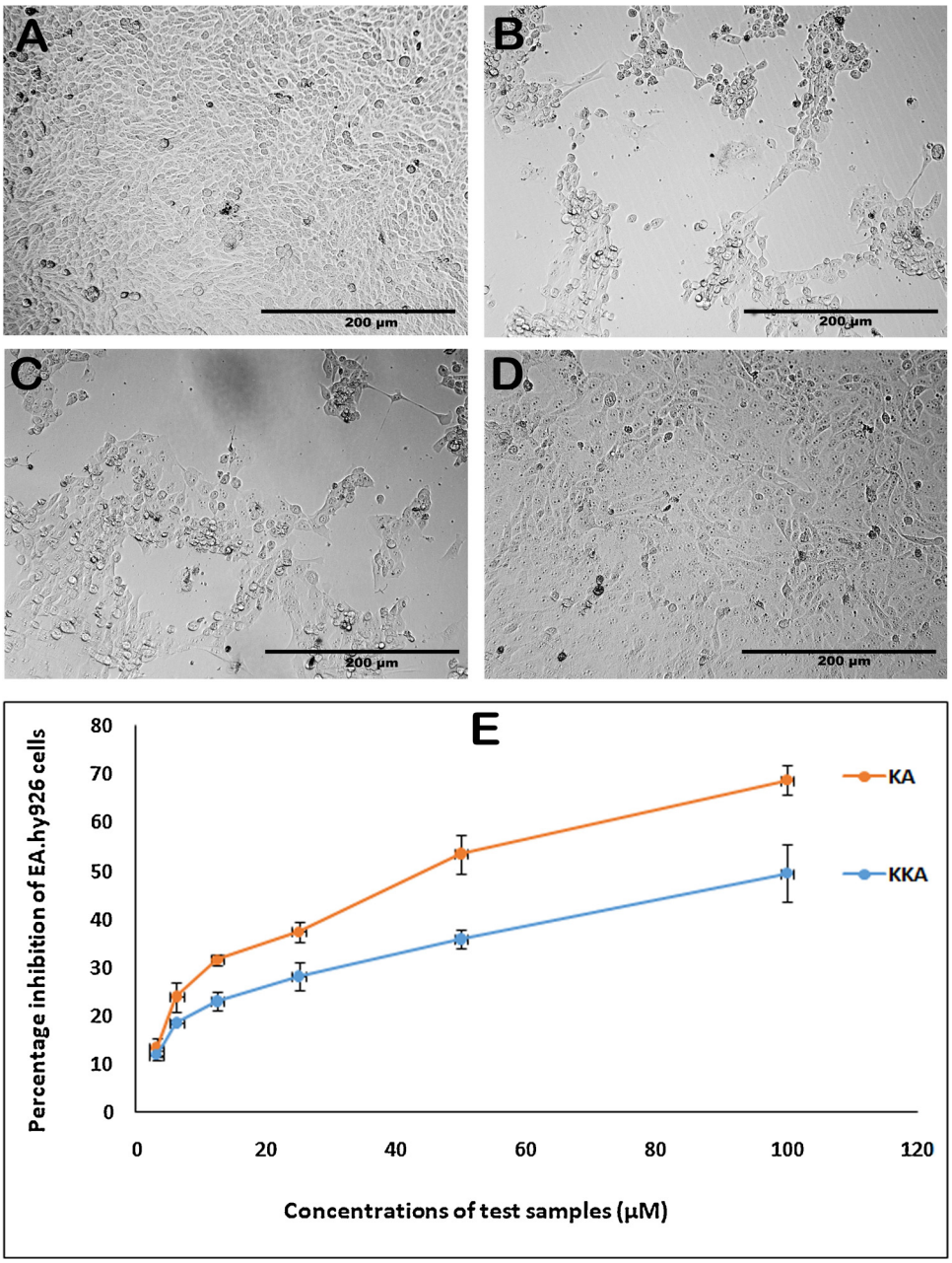


Fig. 4. Anti-proliferative effect of KKA on EA.hy926 cells.
A) Photomicrographic image of untreated (negative control) EA.hy926 cells shows confluent monolayer.
B) Photomicrographic image of the cells treated with Betulinic Acid (50 µM) demonstrates significant cytotoxic effect with reduced doubling population.
C) Photomicrographic image of the cells treated with koetjapic acid (50 µM) demonstrated strong cytotoxicity against the endothelial cells.
D) Photomicrographic image of the cells treated with KKA (50 µM) demonstrated poor cytotoxic effect as the cellular population was not significantly affect when compared to that of the negative control.
E) Graphical representation of dose-dependent effect of KKA and KA on viability of EA.hy926 cell. Values are expressed in mean ± SD of three independent experiments (n = 3).

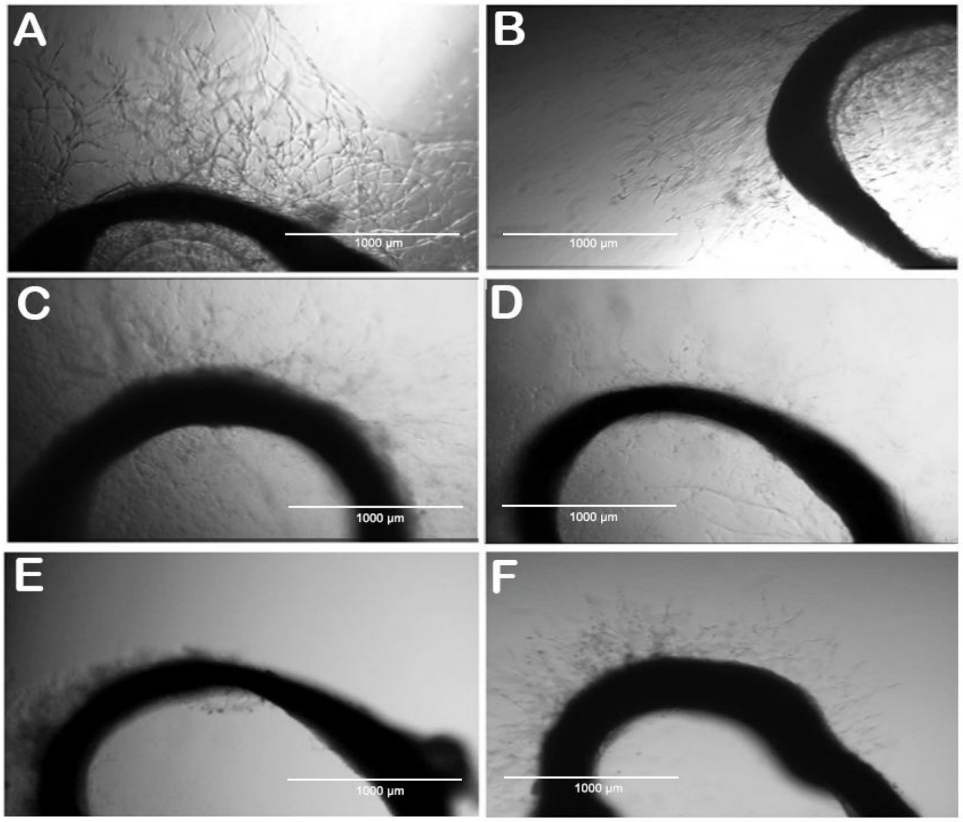
3. Results and discussion

3.1. Method validation

KKA was well-separated from potential interfering compounds in rat plasma under the described chromatographic conditions. Chromatographic peak was identified with the aid of a pure standard of KKA based on retention time. The chromatogram of blank rat plasma and plasma spiked with BA (as an internal standard) and KKA are shown in Fig. 1A and B, respectively. The retention times (R_t) for BA and KKA were recorded at 8.3 and 11.7 min, respectively.

The linearity of detector response was assessed for extracted plasma samples. Linearity was evaluated by determining a series of six concentrations of the KKA standard solution in three replicates. A linear regression equation and correlation coefficient were established from the graph by plotting the plotting concentration versus corrected response (peak area of KKA to the peak area of BA). The standard calibration curve developed for KKA in rat plasma is given in the Fig. 1C. The results showed that the curve obtained for KKA in plasma was linear in the concentration range of 0.3125 − 10 µg/mL (y = 0.5298x + 0.1972, R^2 = 0.999). The LLOD and LLOQ were 0.10 and 0.31 µg/mL, respectively (Table 1).

Deproteinization with methanol gave a high percentage recovery ranging from 95.60 % to 99.34 %, with %RSD 0.87–2.10 indicating a good accuracy of the method (Table 2). Thus, implying that deproteinization of the plasma with methanol did not result in any substantial loss of the chemical constituents. The peak area ratio of KKA over the internal standard (BA) peak area was used to calculate the %RSD of KKA. Intra-day and inter-day precisions were estimated for the 6 replicates of standard KKA samples at six different concentrations in plasma. Intra-day and inter-day precision data are presented in Table 3. The results revealed that, quite acceptable precision values in the range of 91.69–102.70% were obtained with the method as the mean %RSD for KKA was < 2 %. These results indicate that the method is reliable, repeatable and reproducible. Overall, the above results demonstrated a rapid, simple and sensitive HPLC analytical method for KKA analysis in



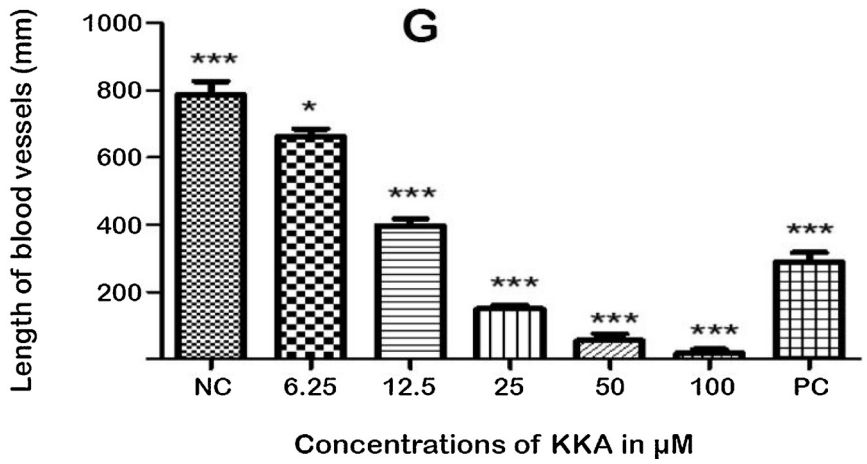


Fig. 5. Effect of KKA on sprouting of microvessels in rat aortic explants. The aortic rings were photographed at 4× magnification, after five days of treatment.
A) Rat aortic ring treated with 0.1 % DMSO (negative control) shows formation of prominent microvessels.
B) Rat aortic ring treated with 12.5 µM KKA.
C) Rat aortic ring treated with 25 µM KKA.
D) Rat aortic ring treated with 50 µM KKA.
E) Rat aortic ring treated with 100 µM KKA.
F) Rat aortic ring treated with 24 µM betulinic acid.
G) Graphical representation of dose-dependent inhibitory effect on spouting of microvessels in rat arotic explant. Values shown are mean ± SD of three independent experiments (n = 3). * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$. NC = negative control; PC = positive control.

rat plasma samples.

3.2. Bioavailability of KKA in rats

Following single dose (50 mg/kg body weight) administration of KKA in the rats, no adverse reactions were observed in both groups (intravenous and oral) of rats. The validated HPLC method was employed to study the pharmacokinetic behaviors of KKA in rat plasma after IV and oral administration of KKA. As shown in Fig. 2, KKA was well-separated from interference components in plasma by the developed HPLC method after oral (Fig. 2A) and intravenous (Fig. 2B) administrations.

The curves of mean plasma concentration versus time profiles of KKA after intravenous and oral administration of KKA are depicted in Fig. 3A and B, respectively. Results were derived from the mean of calculated concentration from three replicate injections. The intravenous and oral pharmacokinetic parameters of KKA were calculated and presented in Tables 4 & 5 . Percentage of the oral bioavailability was calculated by using $AUC_{oral/dose}$ divided by $AUC_{iv/dose}$.

After 50 mg/kg intravenous injection, the concentration of KKA reached a maximum of 10.8 ± 2.3 µg/mL, and $t_{1/2}$ was 0.54 ± 0.02 h. However, after the oral administration of KKA (50 mg/kg) was absorbed slowly and reached C_{max} 7.24 ± 0.36 µg/mL at 2.89 ± 0.12 h, and $t_{1/2}$ was 1.46 ± 0.03 h. Furthermore, following intravenous administration, KKA showed a gradual decline in its plasma concentrations (Fig. 3A). The estimated distribution volume (V_d) of KKA was 0.15 ± 0.01 L/kg h. This suggests that KKA was less distributed into the tissue compartment (Table 4). However, KKA appeared to be clear from the body slowly with its small mean clearance value 0.20 ± 0.01. In addition, KKA showed a small half-life (0.54 ± 0.02). Therefore, it could be suggested that KKA was less distributed into the tissue and retained in the blood compartment for a shorter time. After oral administration, an increase of the plasma concentration of KKA was observed in the plasma samples (Fig. 3B). This may indicate a gradually

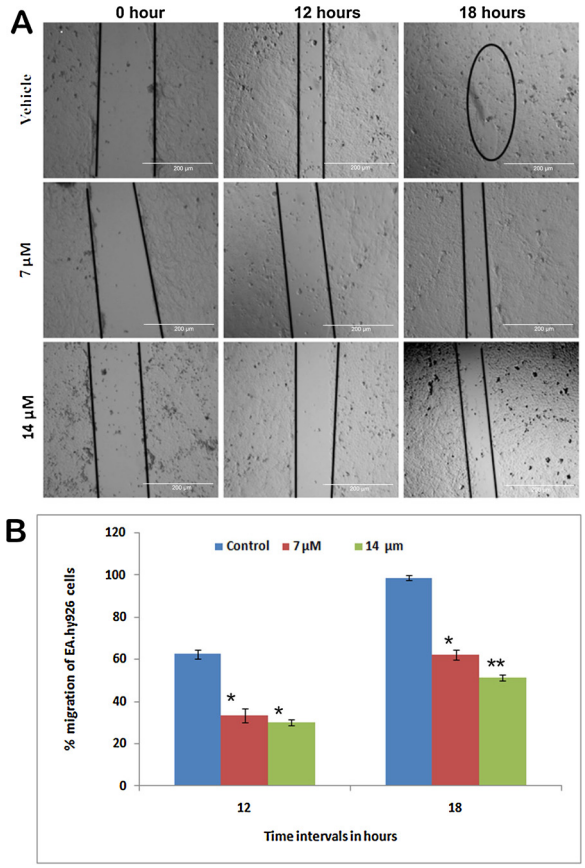


Fig. 6. Effect of KKA on migration of EA.hy926 cells.
A) In untreated group, the endothelial cells the migrated successfully which resulted in complete closure of wound after 18 h, whereas in KKA treated group, the wound remained open even after 18 h incubation. KKA (7 µM) caused significant inhibition of endothelial cell migration. At a concentration of 14 µg/mL, KKA caused severe dislodgement of monolayer of endothelial cells with complete inhibition of migration of the cells.
B) Graphical representation of the time and dose-dependent inhibitory effect of KKA on migration of EA.hy926 cells (values are presented in mean ± S.E.M, $P < 0.005$, $n = 6$).

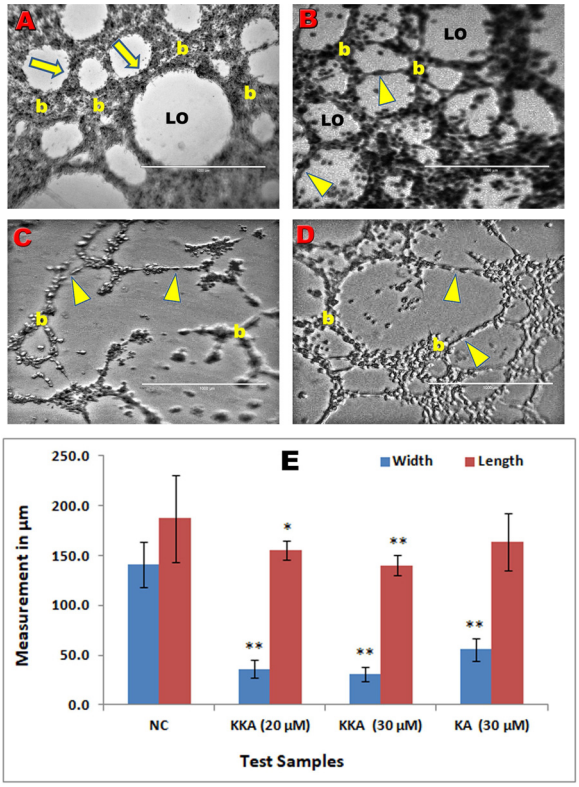


Fig. 7. Effect of KKA in tube formation assay. EA.hy926 (2×10^4 cells/well) were plated on Matrigel precoated 96-well plates and treated with different concentrations of KKA for 24 h. Phase contrast micrographs showing the effects of KKA on differentiation of the cells.
A) Photomicrographic image represents negative control, shows the endothelial cells grown on the 3-dimentional matrigel media differentiated into tube formation. The picture clearly shows prominently thick-cell covered areas (brackets), tubes (arrows), loops (Lo) and branching points (b).
B) Photomicrograph shows the effect of treatment of KKA (20 µM) caused weakening of the bridges in tube-like structure (arrow head) and reduced density of branching points (b) demonstrating moderate anti-angiogenic effect.
C) Treatment with KKA (30 µM) shows significant abrogation of the network structure (arrow head) of the endothelial cells which is evident through the loss of cell covered area (brackets), thinned tubes (arrow head), and feeble branching points (b).
D) Treatment with koetjapic acid (30 µM) shows weakening of the network structure (arrow head) of the endothelial cells with 68.92 ± 2.3 % inhibition.
E) Graphical depiction of dose-dependent inhibitory effect of KKA on the on the height and width of capillary-tube like structures of HUVECs. Values are expressed as mean ± S.E.M, three independent experiments (n = 3).* = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$. KA = koetjapic acid; KKA = potassium koetjapate.

increased absorption of KKA into the blood. The results also indicate that KKA stayed in the body for a relatively long time, as the C_{max} 7.24 ± 0.36 µg/mL in 2.89 ± 0.12 h. The AUC_0 values of KKA for the intravenous group and the oral group was 83.87 ± 4.71 and 26.92 ± 0.01 µg L/mL, respectively. A significantly high AUC_0 value of KKA was obtained in the oral group which indicates a great absorption and utility of this compound in rat plasma. These results indicated that, after oral administration, KKA was absorbed and eliminated slowly. Further, the results imply that after oral administration, KKA was retained in the body for a longer period than that of the intravenous administration. The mean residence time (MRT) for the intravenous group and the oral group were 1.43 ± 0.11 and 2.51 ± 0.42 h, respectively. Thus, the absolute bioavailability of KKA by oral route was 32.09 ± 3.06 %, and this reveals that the KKA is readily bioavailable *via* oral route.

It is reported that more than 50 % of oral therapeutic agents were used in the form of salts [14]. A study showed that salt formation is the most common and effective method for acidic and basic drugs to enhance solubility, rate of bioabsorption and bioefficacy of such drugs [15]. This fact indicates that the salification (salt formation) of a drug molecule is a crucial procedure in development of a drug formulation. The chief objective of the current study was to investigate the bioavailability and bioefficacy of potassium koetjapate. Pharmacokinetic results demonstrated that KKA was readily bioavailable and stayed in the body for a relatively long time after oral administration. It is reported that most of the triterpenoids show poor absorption and limited oral bioavailability [16,17]. However, the present study exhibited that salification of the triterpenoid, koetjapic acid, enhances its bioavailability.

3.3. Anti-angiogenic effect of KKA

3.3.1. KKA inhibits the proliferation of endothelial cells

The effect of different concentrations of KKA and KA on the viability of EA.hy926 cells were illustrated in Fig. 4. The photomicrographic images of untreated EA.hy926 cells (negative control) show full confluent monolayer (Fig. 4A), whereas the positive control, betulinic acid (50 µM) and koetjapic acid (50 µM) demonstrated significant cytotoxicity against the cellular proliferation (Fig. 4B and C, respectively). Interestingly, KKA (50 µM) displayed poor cytotoxic effect against the

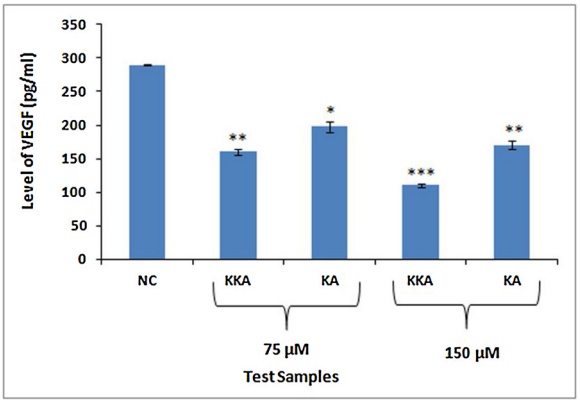


Fig. 8. Effect of KKA on the release of VEGF-A from EA.hy926 cells. Significant decrease in the secretion of VEGF was detected in all the treatment groups. Values shown are mean ± SD of three independent experiments (n = 3). ns = * = $p < 0.05$, ** = $p < 0.001$ and *** = $p < 0.001$, respectively.

endothelial cells as the population of the cells had not been affected by the treatment (Fig. 4D). However, all the tested compounds caused reduction in the percentage of EA.hy926 viable cells gradually in a dose dependent manner compared to the vehicle treated group (Fig. 4E). The IC_{50} values of KA and KKA towards EA.hy926 cells were calculated to be 71.2 µM and 142 µM, respectively.

3.3.2. KKA suppresses sprouting of blood vessels from rat aorta

The inhibitory effect of KKA on blood vessel outgrowth from the rat aortic explants is displayed in Fig. 5. The negative control group, which shows the prominent sprouting of microvessels from the aortic ring is presented in Fig. 5A. KKA caused significant loss of the extent of length and number of the microvessels in the isolated aortic explants (Fig. 5B to E). The anti-neovascularization effect of KKA can be compared with that of the positive control, betulinic acid (100 µM) which caused significant inhibition in formation of blood vessels in aortic rings (Fig. 5F). The graphical presentation of comparative and dose-dependent analysis of sprouting of blood vessels in negative control (0.1 % DMSO), positive control group (betulinic acid) and KKA treated groups (Fig. 5G). According to percentage decrease in blood vessel outgrowth treated with different doses of KKA, the IC_{50} of KKA is estimated as 18.4 ± 4.2 µM.

3.3.3. KKA inhibits migration of endothelial cells

Inhibitory effect of KKA on the motility and migration of EA.hy926 cells is displayed in Fig. 6. A scratch was created in the monolayer of EA.hy926 cells. The wound (scratch) of untreated cells was closed completely within 18 h (Fig. 6A). Whereas, the treatment groups show incomplete wound closure even after 18 h of incubation, as KKA treatment caused significant ($p < 0.05$) inhibition of cellular migration. Wound closure percentages after 12 h were calculated to be 33.41 % and 30.23 % at 7 and 14 µM of KKA, respectively. Whereas, the wound closure was reached to be 62.43 % and 51.3 % after 18 h of treatment with 7 and 14 µM of KKA, respectively. The significant inhibition of migration of EA.hy926 cells by KKA can be observed in the graphical presentation (Fig. 6B) which depicts the reduced percentage migration of the cells across the artificially created wound in comparison with negative control group (Fig. 6B).

3.3.4. KKA inhibits differentiation of endothelial cells on Matrigel Matrix

Treatment of EA.hy926 cells with KA inhibited the growth factor induced differentiation in a dose-dependent manner. Because of the growth factor influenced differentiation, a well prominent tube-like network was formed in the endothelial cells (Fig. 7A). Whereas, treatment with KKA at 20 µM concentrations (Fig. 7B), affected the tube-like structure and decreased the length and width of the network through which the endothelial cells connected and established the vasculature like structure. At 30 µM, KKA completely abrogated endothelial tube formation (Fig. 7C). Koetjapic acid (KA) which was used as reference (Fig. 7D) in this study showed less activity (68.92 ± 2.3 % at 30 µM) as compared to the equivalent dose of KKA ($p < 0.05$). The dose dependent effects of KKA on width and length of endothelial cell tube-network is depicted in Fig. 7E which shows significant inhibition of EA.hy926 tube formation by KA and KKA as compared to negative control ($p < 0.05$).

3.3.5. KKA blocks production of vascular endothelial growth factor (VEGF)

The concentration of VEGF in untreated and treated (KKA and koetjapic acid) endothelial cells (EA.hy926) is depicted in Fig. 8. The results show a significant decrease ($p < 0.05$) in the amount of VEGF observed in koetjapic acid and KKA treated cells when compared with untreated cells. Concentration of VEGF in the control group (distilled water) was 89 ± 2.21 pg/mL. Whereas, concentrations of VEGF in cells treated with 75 and 150 µM of KKA, and 75 and 150 µM of KA are 151.2 ± 3.9 ($p < 0.001$), 102.34 ± 3.4 ($p < 0.001$), 189.2 ± 4.3 ($p < 0.05$), and 184 ± 5.4 pg/mL ($p < 0.001$), respectively. When comparison is made between treatment groups, KKA shows significantly more potent ($p < 0.05$) anti-VEGF activity at higher concentration (150 µm/mL) compared to koetjapic acid at higher concentration (150 µm/mL) which indicates that KKA has better efficacy in inhibition of VEGF activity compared to KA which is used as reference in this study.

3.3.6. KKA inhibits matrigel induced vasculature in nude mice

Overall results of Matrigel plug assay illustrating the antiangiogenic activity is depicted in Fig. 9. Matrigel was implanted in nude mice subcutaneously and then allowed 21 days to observe the formation of neovascularization. Mice bearing the Matrigel plug that represents negative control group (Fig. 9A). The Matrigel plug harvested from the negative control group is depicted in Fig. 9B. The morphological studies of the isolated Matrigel plug from the negative control group revealed blood vessels formed most prominently, whereas KKA treatment caused significant (*** = $p < 0.001$) reduction in the formation of new blood vessels. The animal and the Matrigel plug corresponding to the KKA treatment with 50, 100 and 200 mg/kg body weight are represented in Fig. 9C–D, E–F and G–H, respectively. Treatment with standard drug, Imatinib (100 mg/kg) has shown significant (*** = $p < 0.01$) anti-neovascularization in the animals bearing Matrigel (Fig. 9I), as the isolated Matrigel plug (Fig. 9J) showed reduced formation of blood vessels. The Matrigel plug harvested from higher concentration of KKA (200 mg/kg)-treated animals revealed drastic reduction of blood vessels in the Matrigel implant. The findings were also confirmed in the H&E stained cross-sections of the excised Matrigel implants (Fig. 10). Microtome section of the Matrigel from the negative control group (Fig. 10A) displays high vascularization (circles) and more proliferation of HCT-116 cells compared to the other KKA treated groups (Fig. 10B and C). The anti-neovascularization effect of KKA is comparable with that of the standard reference, imatinib tested at 100 mg/kg (Fig. 10D).

The average volume of Matrigel plug harvested from the representative animals is shown in Fig. 10E. The plugs excised from the negative control group demonstrated larger size volume of 633.9 ± 23 cm^3. Whereas, KKA showed a dose-dependent effect on the plug volume with 304.0 ± 16 cm^3, 220.5 ± 39 cm^3, and 200.7 ± 11 cm^3 for the doses 50, 100 and 200 mg/kg, respectively. The effect of KKA was comparable with the positive control Imatinib (100 mg/kg) which exhibited 232.8 ± 11 cm^3 plug volume. The average blood vessel counts taken from 10 microscopic fields (low magnification power) in Matrigel sections from representative groups are displayed in a graphical representation in Fig. 10F. The graph shows that the average blood vessel count in the negative control group was 109 ± 5, whereas treatment with KKA has significantly ($P < 0.01$) inhibited formation of new blood vessels in the Matrigel implants, as the total mean counts of blood vessels were 46 ± 3 and 19 ± 2 and 11 ± 5 at concentrations of 50,

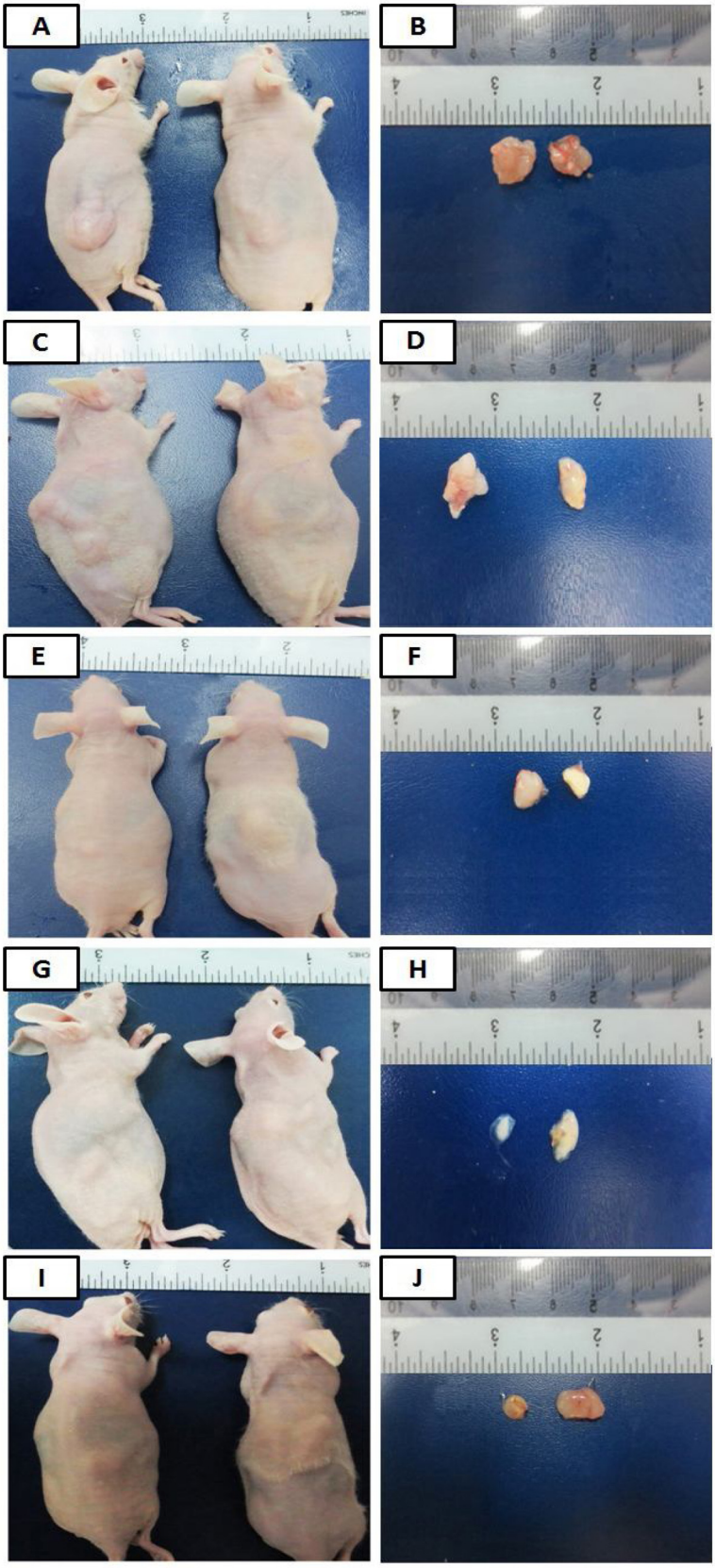


Fig. 9. *In vivo* anti-angiogenic effects of KKA was determined using Matrigel plug model in athymic nude mice. KKA exhibited strong inhibitory effect on vascularization in Matrigel plugs implanted in nude mice when compared to negative control treated group.
A) Animals represent negative control group.
B) Matrigel plug isolated from the negative control group.
C) Animals treated with KKA (50 mg/kg).
D) Matrigel plug isolated from KKA (50 mg/kg) treated group.
E) Animals treated with KKA (100 mg/kg).
F) Matrigel plug isolated from KKA (100 mg/kg) treated group.
G) Animals treated with KKA (200 mg/kg).
H) Matrigel plug isolated from KKA (200 mg/kg) treated group.
I) Animals treated with imatinib (100 mg/kg).
J) Matrigel plug isolated from imatinib (100 mg/kg) treated group.

100 and 200 mg/kg, respectively. The blood vessel count in Matrigel implants from the mice treated with imatinib at 100 mg/kg was calculated to be 20 ± 4.

In the present study, antiangiogenic potentials of KKA were investigated with a series of *in vitro* and *in vivo* assays. Results of rat aortic ring assay revealed a strong inhibitory effect of KKA on neovascularization in aortic explants. In order to examine whether the observed effect on rat aortic ring assay was because of inhibition of

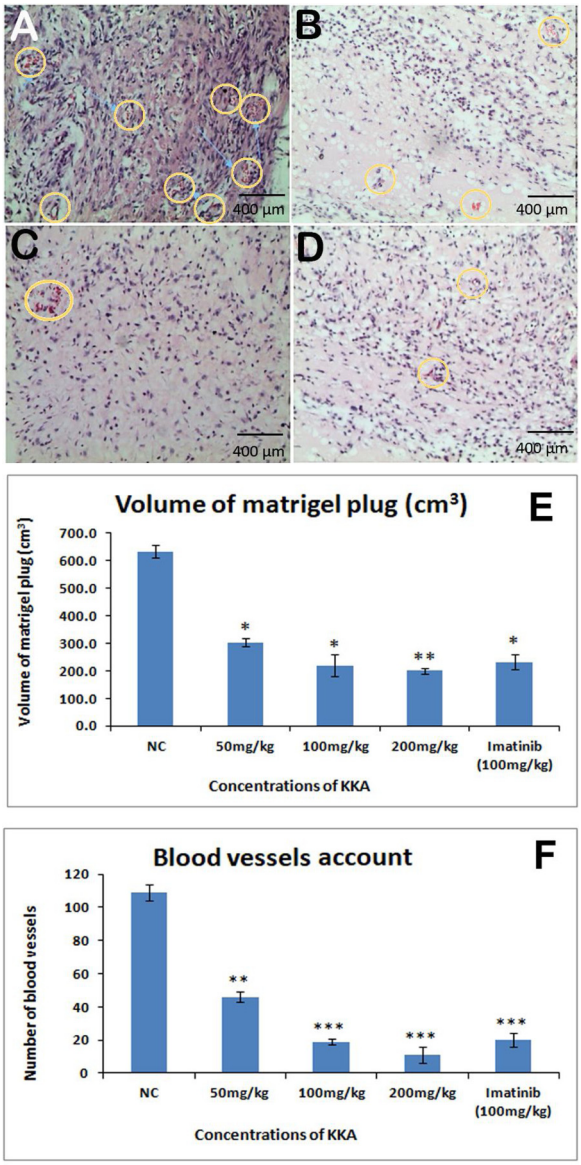


Fig. 10. H&E stained cross-sections taken from Matrigel plugs implanted subcutaneously in representative groups of animals.
A) Section from negative control group shows prompt and well-developed blood vessels (indicated with circles).
B) Section from imatinib treated group (positive control) demonstrates less blood vessels when compared to the negative control.
C) Sections from KKA (100 mg/kg) treated group shows moderate inhibition of blood vessel formation in Matrigel plugs.
D) Sections from KKA (200 mg/kg) treated group shows pronounced inhibition of blood vessel formation in Matrigel plugs.
E) Graphical representation of the effect of KKA on the volume of Matrigel plug. (*** = $p < 0.001$, n = 6, values are mean ± SEM of 10 low power microscopic fields). NC = negative control; KKA = potassium koetjapate. Imatinib is used as positive control.
F) Graphical representation of the effect of KKA on the mean blood vessel count (average of 10 microscopic fields) in Matrigel sections. (*** = $p < 0.001$, n = 10, values are mean ± SEM of 10 low power microscopic fields). NC = negative control; KKA = potassium koetjapate. Imatinib is used as positive control.

endothelial cells proliferation or not, the MTT assay was conducted on EA.hy926 cells. KKA displayed a very poor cytotoxic effect on the human endothelial cells with a high IC_{50} value (142 µM) which demonstrates that KA is not a cytotoxic compound against EA.hy926 cells. Interestingly, KKA has shown reduced cytotoxicity on EA.hy926 cells compared to the parent molecule koetjapic acid which has shown significant cytotoxic effect with IC_{50} 71.2 µM. Consequently, the results of rat aortic ring assay and the MTT assay on EA.hy926 cell imply that the anti-angiogenic effect presented by KKA is not because of the cytotoxic essence of the compound, but may be more related to inhibition of endothelial functions such as motility, migration, differentiation and/or inhibition of VEGF signaling. To find out the mechanism of anti-angiogenesis of KKA, its effect was examined on several major steps of angiogenesis, such as migration, differentiation and VEGF expression in human endothelial cells. In addition, the effect of KKA was also studied on formation of Matrigel-induced vasculature in nude mice. The results show that KA significantly suppressed all the mentioned steps. The critical cascade of angiogenesis is the degeneration of extracellular matrix and vascular basement membrane which paves the way for endothelial cells to migrate into the perivascular space in the direction of angiogenic stimuli, mostly VEGF. Endothelial cells follow chiefly the chemotaxic migration towards the chemoattractants such as VEGF [18]. In the present study, KKA displays significant improvement in inhibition of VEGF activity compared to KA, which was used as reference in this study. The findings of the present study indicate a significant enhancement in antiangiogenic efficacy of KKA in comparison with its parent compound koetjapic acid. KKA has shown antiangiogenic properties by stopping sprouting of rat aortic microvessels, and migration and differentiation of endothelial cells. In addition, KKA displays antiangiogenic activity by suppressing VEGF production. Previous studies have shown that KA induces cytotoxicity *via* apoptotic pathway by inhibiting the Wnt and HIF-1α genes activities [1]. In addition, KA has also demonstrated remarkable antiangiogenic effects *via* VEGF inhibition [19]. Several studies have reported that Wnt and HIF-1α genes have been strongly coupled with VEGF inhibition [20,21]. A growing number of anticancer agents have been shown to inhibit VEGF pathway *via* blocking hypoxia inducible factor (HIF) and Wnt genes activity [22]. Based on these facts, the mechanism of action for KA and KKA can be established that involves suppression of HIF-1α and Wnt genes activity which in turn blocks VEGF pathway and subsequently inhibits angiogenesis. Findings of the present study showed that potassium koetjapate (KKA) has shown improved cytotoxic as well as antiangiogenic effects compared to koetjapic acid (KA). The improved efficacy of KKA can be due to the improved solubility and bioavailability.

4. Conclusion

Based on the outcome of the current work, it can be concluded that a simple and sensitive HPLC method has been successfully applied to determine the pharmacokinetic characteristics of potassium koetjapate (KKA) in rats. The results indicated that KKA was readily absorbed into the rat blood upon oral administration. Furthermore, the anti-angiogenic studies revealed remarkable improvement in the efficacy of KKA compared to the parent compound, koetjapic acid. KKA suppressed angiogenesis by inhibiting endothelial functions and VEGF expression. The enhanced bioefficacy of KKA could probably be attributed to its enhanced solubility and bioavailability. Further preclinical and clinical studies are warranted to develop KKA as a promising therapeutic agent against angiogenesis related disorders.

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Declaration of Competing Interest

All authors of the manuscript have declared that there is no conflict of interest.

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