Pharmacokinetics and antiangiogenic studies of potassium koetjapate in rats

Seyedeh F. Jafari
Fouad Saleih R. Al-Suede
Ashwaq H.S. Yehya
Mohamed B.K. Ahamed
Armaghan Shafaei  
*Edith Cowan University*

*See next page for additional authors*

Follow this and additional works at: [https://ro.ecu.edu.au/ecuworkspost2013](https://ro.ecu.edu.au/ecuworkspost2013)

Part of the Pharmacology Commons

This Journal Article is posted at Research Online.  
Authors
Seyedeh F. Jafari, Fouad Saleih R. Al-Suede, Ashwaq H.S. Yehya, Mohamed B.K. Ahamed, Armaghan Shafaei, Muhammad Asif, Yasser M. Tabana, Amin M.S.A. Majid, and Hussein M. Baharetha
Original article

Pharmacokinetics and antiangiogenic studies of potassium koetjapate in rats

Seyedeh F. Jafari, Fouad Saleih R. Al-Suede, Ashwaq H.S. Yehya, Mohamed B.K. Ahamed, Armaghan Shafaei, Muhammad Asif, Yasser M. Tabana, Amin M.S.A. Majid, Hussein M. Baharethag

a EMAN Research and Testing Laboratory, Department of Pharmacology, School of Pharmaceutical Sciences, Universiti Sains Malaysia, Penang, Malaysia
b EMAN Biodiscoveries Sdn. Bhd., Halal Park, 08000, Sungai Petani, Kedah, Malaysia
c Centre for Integrative Metabolomics and Computational Biology, School of Sciences, Edith Cowan University Joondalup, WA, 6027, Australia
d Department of Pharmacy, The Islamia University of Bahawalpur, 63100, Pakistan
e Faculty of Pharmacy & Pharmaceutical Sciences, University of Alberta, 116 St & 85 Ave, Edmonton, AB, T6G 2R3, Canada
f John Curtin School of Medical Research, College of Medicine, Australian National University, Australia
g Department of Pharmacy, College of Medicine and Health Sciences, Hadhramout University, Mukalla, Hadhramout, Yemen

ARTICLE INFO

Keywords:
Koetjapic acid
Potassium koetjapate
Sandoricum koetjape
Bioavailability
HPLC
Angiogenesis

ABSTRACT

Purpose: Koetjapic acid is an active compound of a traditional medicinal plant, Sandorica 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 Tmax 2.89 ± 0.12 h, Cmax 7.24 ± 0.36 μg/mL and T1/2 1.46 ± 0.03 h. The pharmacological results showed that KKA significantly suppressed sprouting of microvessels in rat aorta with IC50 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.

Sandorica 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,
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].

2.3.2. Determination of pharmacokinetic parameters

The pharmacokinetic parameters estimated from the data of IV administration were, area under plasma concentration-time curve (AUC0→∞), elimination rate constant (Ke), biological half-life (t1/2), volume of distribution (Vd), and clearance (CL). The value of AUC0→∞ (μg h/mL) was determined by adding the area from time zero to the last sampling time (AUCl) and to the area from last sampling time to infinity (AUCl→∞). The Ke (h−1) was calculated using the relationship, t1/2 = ln 2/Ke while Vd = Ke × AUCl→∞. Clearance CL (L/kg h) was calculated from the relationship, CL = dose/AUC0→∞. Whereas from the data of oral administration, area under plasma concentration-time curve AUC0→24 (μg h/mL), maximum concentration (Cmax) and time to reach Tmax to maximum concentration were calculated. The percentage of absolute bioavailability of the standard compounds was estimated from the following equation [5]:

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 (Rt) values 8.3 and 11.7 min, respectively. Standard calibration curve of potassium koetjapate (KKA) in rat plasma depicting the linear regression curve.
% Absolute bioavailability (F) = (AUC for oral/AUC for IV) × (IV dose/oral dose) × 100

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>LOD (μg/mL)</th>
<th>LOQ (μg/mL)</th>
<th>Linearity range (μg/mL)</th>
<th>Equation</th>
<th>R² Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>KA</td>
<td>0.10</td>
<td>0.31</td>
<td>0.3125 – 10</td>
<td>y = 0.5298x + 0.1972</td>
<td>0.999</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Name</th>
<th>Concentration (μg/mL)</th>
<th>Recovery (Mean ± RSD (%))</th>
</tr>
</thead>
<tbody>
<tr>
<td>KA</td>
<td>0.3125</td>
<td>96.41 ± 0.87</td>
</tr>
<tr>
<td></td>
<td>0.625</td>
<td>96.58 ± 1.34</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>97.97 ± 1.56</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>99.34 ± 2.10</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>95.60 ± 1.64</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>98.82 ± 1.39</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration (μg/mL)</th>
<th>Within Day</th>
<th>Between day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Accuracy (% of true value)</td>
<td>RSD (%)</td>
<td>Accuracy (% of true value)</td>
</tr>
<tr>
<td>KA</td>
<td>0.3125</td>
<td>102.70</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>0.625</td>
<td>96.79</td>
<td>1.87</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>92.33</td>
<td>1.07</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>96.45</td>
<td>1.79</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>97.95</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>101.98</td>
<td>2.00</td>
</tr>
</tbody>
</table>

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 % CO₂, 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% CO₂. Then EA.hy926 were trypsinized and seeded (3 × 10⁴ cells per
fourth day. On the next day, aortic rings were imaged using an inverted light microscope at 4× magnification. The capillary-like structures were visualized and imaged under light microscopy at 20× and 40× 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:

\[
\text{Blood vessels inhibition} = \left(1 - \frac{A_0}{A}\right) \times 100
\]

In which, \(A_0\) = distance of blood vessels growth in treated rings with KKA in \(\mu\)m, \(A\) = distance of blood vessels growth in the control in \(\mu\)m.

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 \(\mu\)M of KKA and 75 \(\mu\)M and 150 \(\mu\)M 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 Tetrathymethylbenzidine substrate solution was pipetted to the wells. The Stop Solution (H2SO4) 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; \((y = 0.0099 \times 0.6137, \ R^2 = 0.996)\).

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 \(\mu\)l of Matrigel appended with 100 \(\mu\)l of HCT-116 cells, (1 \(\times\) 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 paraformaldehyde, dehydrated and cut. The samples 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].

<table>
<thead>
<tr>
<th>Table 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pharmacokinetic parameters of koetjapic acid (KA) in rat plasma after intravenous administration (n = 6).</td>
</tr>
<tr>
<td>Data</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>AUC(24−h)</td>
</tr>
<tr>
<td>(K_e)</td>
</tr>
<tr>
<td>(t_{1/2})</td>
</tr>
<tr>
<td>(V_d)</td>
</tr>
<tr>
<td>CL</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pharmacokinetic parameters of koetjapic acid (KA) in rat plasma after oral administration (n = 6).</td>
</tr>
<tr>
<td>Data</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>(C_{max})</td>
</tr>
<tr>
<td>(T_{max})</td>
</tr>
<tr>
<td>Oral bioavailability</td>
</tr>
</tbody>
</table>
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 (Rt) 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 \(0.3125 \text{ to } 10 \mu g/mL\) (\(y = 0.5298x + 0.1972, R^2 = 0.999\)). The LOD and LOQ were 0.10 and 0.31 \(\mu g/mL\), 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 precision 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 rat plasma.
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 AUCoral/dose divided by AUCiv/dose.

After 50 mg/kg intravenous injection, the concentration of KKA reached a maximum of 10.8 ± 2.3 μg/mL, and t1/2 was 0.54 ± 0.02 h. However, after the oral administration of KKA (50 mg/kg) was absorbed slowly and reached Cmax 7.24 ± 0.36 μg/mL at 2.89 ± 0.12 h, and t1/2 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 (Vd) 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 cleared 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
This fact indicates that the sali
oral route.

via

32.09 ± 3.06 %, and this reveals that the KKA is readily bioavailable
spectively. Thus, the absolute bioavailability of KKA by oral route was

hanced solubility, rate of bioabsorption and bioef
in the form of salts [14]. A study showed that salt formation is the
most common and ef
efi
ency of such drugs

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 en-
hance solubility, rate of bioabsorption and bioef
cficacy of such drugs

The chief objective of the current study was to investigate the bio-
availability and bioef
cficacy 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 re-
ported 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 bioavail-
nility.

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). In-
ferently, KKA (50 μM) displayed poor cytotoxic effect against the

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 sig-
nificant 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-dimensional matrigel media differentiated into tube for-
mation. 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 ex-
pressed 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.
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 IC₅₀ 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 displayed 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 IC₅₀ 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. 9D), 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). Micromorphometric 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 cm³. Whereas, KKA showed a dose-dependent effect on the plug volume with 304.0 ± 16 cm³, 220.5 ± 39 cm³, and 200.7 ± 11 cm³ 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 cm³ 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,
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. 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.
Interestingly, KKA has shown reduced cytotoxicity on EA.hy926 cells which indicates that KA is not a cytotoxic compound against EA.hy926 cells. n = 10, values are mean ± SEM of 10 low power microscopic fields. NC = negative control; KKA = potassium koetjapate. Imatinib is used as positive control.

**Declaration of Competing Interest**

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

The authors thankfully acknowledge Ministry of Education (MOE) Malaysia for the FRGS grant (No. 203/PFARMASI/6711260). The authors also acknowledge University Sains Malaysia for the Research University Team (RUT) grant number 1001/PFARMASI/851001.

References