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Antimalarial activity of selected Ethiopian medicinal plants in mice

[Actividad antipalúdica de plantas medicinales etíopes seleccionadas en ratones]

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Abstract

Context: Parasites are the leading killers in subtropical areas of which malaria took the lion share from protozoan diseases. Measuring the impact of antimalarial drug resistance is difficult, and the impact may not be recognized until it is severe, especially in high transmission areas.

Aims: To evaluate the *in vivo* antimalarial activities of hydroalcoholic extracts of the roots of *Piper capense* and *Adhatoda schimperiana*, against *Plasmodium berghei* in mice.

Methods: Four-day suppressive and curative test animal models were used to explore the antimalarial activities of the plants. 200, 400, and 600 mg/kg of each plant extract was administered to check the activities *versus* vehicle administered mice. Mean survival time and level of parasitemia were the major variables employed to compare the efficacy *vs.* negative control.

Results: In both models the 400 and 600 mg/kg doses of *Adhatoda schimperiana* and the 600 mg/kg dose *Piper capense*. showed significant parasitemia suppression and increased in mean survival time at p≤0.05. The middle dose of *Piper capense* had a border line inhibition where the extracts were considered active when parasitemia was reduced by ≥ 30%.

Conclusions: The hydroalcoholic extracts of the roots of *Adhatoda schimperiana* and *Piper capense* possess moderate antimalarial activities, which prove its traditional claims. Thus, further studies should be done to isolate the active constituents for future use in the modern drug discovery.

Keywords: *Adhatoda schimperiana*; curative test; four days suppressive test; malaria; *Piper capense*; resistance.

Resumen

Contexto: Los parásitos son los principales asesinos en áreas subtropicales, de los cuales la malaria forma la mayor parte de las enfermedades protozoarias. Medir el impacto de la resistencia a los medicamentos antipalúdicos es difícil y el impacto puede no ser reconocido hasta que sea severo, especialmente en áreas de alta transmisión.

Objetivos: Evaluar la actividad antimalárica *in vivo* de extractos hidroalcohólicos de las raíces de *Piper capense* y *Adhatoda schimperiana* contra *Plasmodium berghei* en ratones.

Métodos: Se utilizaron modelos animales de prueba supresora de cuatro días y curativa para explorar la actividad antimalárica de las plantas. Se administraron 200, 400 y 600 mg/kg de cada extracto para comparar las actividades frente a los ratones administrados con el vehículo. El tiempo medio de supervivencia y el nivel de parasitemia fueron las principales variables empleadas para comparar la eficacia frente al control negativo.

Resultados: En ambos modelos, las dosis de 400 y 600 mg/kg de *Adhatoda schimperiana* y la dosis de 600 mg/kg de *Piper capense* mostraron una significativa supresión de la parasitemia (p≤0.05) y un aumento en el tiempo medio de supervivencia. La dosis media de *Piper capense* tuvo una inhibición en la línea límite donde los extractos se consideraron activos cuando la parasitemia se redujo en ≥30%.

Conclusiones: Los extractos hidroalcohólicos de las raíces de *Adhatoda schimperiana* y *Piper capense* poseen actividad antipalúdica moderada, que corroboran sus usos tradicionales. Por lo tanto, se deben realizar más estudios para aislar los constituyentes activos para el posible descubrimiento de nuevos fármacos.

Palabras Clave: *Adhatoda schimperiana*; malaria; *Piper capense*; prueba curativa; prueba supresora cuatro días; resistencia.

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INTRODUCTION

Malaria continues to be a global health issue despite the huge gains over the past decade. According to the WHO global malaria report, in 2015 the number of malaria cases and deaths were 212 million and 429,000, respectively. Africa continues to make up the larger share of about 90% of both cases and deaths coming from this region (WHO, 2016). The multi-strategy global fight is complicated by many challenges one of which is related to treatment.

Accessibility and resistance are the major determinates of malaria treatment outcome (Getachew et al., 2016). Drug efficacy is one that has been majorly threatened. For example, *Plasmodium falciparum* is resistant to nearly all anti-malarial drugs in current use (Abdulelah et al., 2011). Chloroquine resistance was reported decades ago in Southeast Asia and South America in the 1950s, and Africa in the 1970s. Reports of quinine resistance is also an additional headache to malaria treatment. Currently, artemisinins are the most effective antimalarial agents. They have widely used either alone or in combination to treat and prevent the disease (WHO, 2011). Unfortunately, *P. falciparum* resistance to artemisinins has been confirmed in five countries as of 2015. Particularly in Cambodia high failure rates to four different artemisinin therapies have been detected (WHO, 2016). With the current high use of these drugs, the selective pressure is only going to grow to pose a serious problem to malaria treatment. Hence, for treatment to continue as a viable strategy to meet global targets against malaria, it is paramount that new therapies come into practice.

Traditionally used herbal medicines have been behind many drug discovery successes including anti-malaria agents. Numerous plants traditionally claimed for malaria treatment across the world were proved scientifically for their effectiveness (Abdulelah et al., 2011). Some modern drugs in current clinical practice such as quinine and artemisinins stand as a testament (Ajaiyeoba et al., 2006; Deressa et al., 2010). In addition to being potential starting points for drug discovery efforts, they will also help address accessibility and affordability problems, and in turn, positively affect malaria treatment outcome. Thus, we have selected two plants, *Adhatoda schimperiana* (Hochst.) Nees (Acanthaceae) and *Piper capense* Linn.f. (Piperaceae), commonly used to treat malaria in Ethiopia traditional medicine.

Adhatoda schimperiana, locally called as 'Sensel or Sansali', grows in the highlands of Ethiopia and East Africa. Traditional healers use the root and leaf of this plant for varies ailment such as malaria, rabies, syphilis, leprosy, gonorrhea and measles in Northern Ethiopia. Specifically, the root is used for management of malaria while the leaf is used for relapsing fever (Abebe and Ayehu, 1993; Geyid et al., 2005). Scientifically, the leaves of the plant have been evaluated for its bronchodilator, anti-inflammatory, antimicrobial and antimalarial potential (Assefa et al., 2008; Petros and Melaku, 2012; Abdela et al., 2014).

Piper capense, locally called as 'Timiz', is widely distributed in Southern and Western Ethiopia (Avril, 2008). *P. capense* is traditionally used for diseases like coughs, bacterial, neoplasm, rheumatism, toothache, malarial, psychosis, amoebic flu, hypoglycemic, leishmania. Scientifically, the aerial part of the plant has been evaluated for antiplasmodial and *Trichomonas vaginalis* activities *in vitro* (Fernandes et al., 2008; Chahal et al., 2011).

To our knowledge, no scientific study screened *in vivo* antimalarial activities of *Adhatoda schimperiana* root as well as both *in vitro* and *in vivo* antimalarial activities of *Piper capense* root. Therefore, the present study focused on the evaluation of *in vivo* antimalarial activities by the hydroalcoholic extracts of these two plants against *Plasmodium berghei* in mice.

MATERIAL AND METHODS

Plant materials

Adhatoda schimperiana and *Piper capense* were collected from Jimma and Bonga towns, respectively (7.6739° N, 36.8358° E and 7.2672° N, 36.2468° E). They were taxonomically identified by a botanist, and voucher specimens were deposited (AS001/2014 and PC001/2014) at the Department of Biology, Jimma University. The roots of the plants were dried under shade, powdered and stored in amber glass bottles until extraction. Extraction was carried out through cold maceration as described by Deressa et al. (2010). Finally, both extracts were

concentrated using rotary evaporator and stored in the refrigerator.

Animals

Adult Swiss albino mice of either sex (27-32 g, age of 6-8 weeks) were purchased from the animal house of Ethiopian Health and Nutrition Research Institute (EHNRI). The animals were placed in a standard cage and provided with food and water *ad libitum*. They were randomized into five groups with each group containing six rodents. Among the five groups, three groups received the extract orally at doses of 200, 400 and 600 mg/kg. The remaining two groups were used as controls with one group receiving 10 mg/kg chloroquine and the other 10 mL/kg water.

Animal handling and care were in compliance with international laboratory animal use and care guidelines (ILAR, 1996). Besides, the study was ethically cleared (RPGC/403/2014) by the ethics committee of College of Health Sciences, Jimma University before its commencement.

Experimental protocol

Chloroquine-sensitive *Plasmodium berghei* was used to model malaria in the rodents, which was also obtained from Ethiopian Health and Nutrition Research Institute (EHNRI). First, inoculum donor mice were infected with *Plasmodium berghei* after which they were sacrificed by head blow and blood was collected in a heparinized syringe by heart puncture. Then, inoculums of 0.2 mL blood each with 1 x 10^7 infected red cells were prepared in trisodium citrate medium (Ajaiyeoba et al., 2006). The inoculums were later injected intraperitoneally to induce infection both in the experimental and control groups. Depending on the commencement of test substances administration relative to infection induction, the tests were divided as early infection and established infection. In the early infection activity test, injection of both inoculum and test substances was started concomitantly while in the case of established infection test oral dosing was started 72 h after infection. Once daily oral administration of the extract, vehicle, and standard drug was carried out for 4 days.

Dosing schedule

Negative Control (NG): Water (10 mL/kg) orally administered for four days immediately after inoculum injection (early infection). Water (10 mL/kg) orally administered four days 72 h after of inoculum injection (established infection).

AD200/400/600: *Adhatoda schimperiana* extract (200, 400, and 600 mg/kg b.w.) orally administered for four days immediately after inoculum injection (early infection). *Adhatoda schimperiana* extract (200, 400, and 600 mg/kg b.w.) orally administered for four days 72 h after of inoculum injection (established infection).

PC200/400/600: *Piper capense* extract (200, 400, and 600 mg/kg b.w.) orally administered for four days immediately after inoculum injection (early infection). *Piper capense* extract (200, 400, and 600 mg/kg b.w.) orally administered for four days 72 h after of inoculum injection (established infection).

Positive Control (CQ): Chloroquine (10 mg/kg) orally administered for four days immediately after inoculum injection (early infection). Chloroquine (10 mg/kg) orally administered four days 72 after of inoculum injection (established infection).

Percentage of parasitemia and chemosuppression

After four days of oral administration of test substances, thin smears of blood films were taken from the peripheral blood of the tail of each mouse. The smears were then fixed with methanol and stained with Giemsa to examine the parasitized red blood cells under the microscope (Abdulelah et al., 2011). After microscopic evaluation, the percentage of parasitemia and chemosuppression were calculated as described in Elutioye and Agbedahunsi (2004) and Deressa et al. (2010).

Mean survival time

Apart from measuring the possible effect of the extracts on parasitemia, the study also evaluated their impact on the mean survival time of the rodents used for the early infection test over 30 days post-infection. The mean survival time of a group was computed as a quotient of the sum of survival time of mice in days to the total number of mice in the group. Individual survival values were deter-

mined from death recordings throughout the follow-up period in each group (Mengiste et al., 2012).

Acute oral toxicity test

Oral toxicity test was conducted as per the internationally accepted protocol drawn under OECD guidelines 425 (OECD, 2001). Accordingly, the limit dose of 2 g/kg was administered to the mice and were observed for signs of toxicity over an hour, 4 h, 24 h, and for 14 days.

Statistical analysis

The collected data was analyzed using SPSS version 20. The results were presented as mean ± standard error of the mean (SEM). The analysis was made using one-way ANOVA to compare the means between treatment and control groups. P-value <0.05 was considered significant.

RESULTS

Four-day test

The mice group receiving the hydroalcoholic extract of *Adhatoda schimperiana* at a dose of 600 mg/kg reduced the parasite load by half. Unfortunately, the lower dose suppressed the parasite by nearly less than 20%, which was not statistically significant ($p>0.05$) as compared to the negative control group. The 400 and 600 mg/kg doses of *Piper capense* treated groups had parasitemia load less than the negative control by 30.6 and 48.6%, respectively. The lower dose reduces parasitemia level at a rate equivalent to 50% lower than the parasitemia reducing the capacity of the lower dose of *Adhatoda schimperiana* hydroalcoholic extract (Table 1).

The mean days of survival for the negative control group was 8.2 ± 0.66 days as shown in Table 2. The mice administered with 200 mg/kg dose of *Adhatoda schimperiana* and *Piper capense* had an average survival days almost as long as the negative control group. However, it took 19.6 ± 1.10, 18.0 ± 1.0 and 27.8 ± 0.5 days for the 600 mg/kg dose of *Adhatoda schimperiana* and *Piper capense* and chloroquine-treated groups, respectively. Moreover, the survival difference was significant at $p\leq0.05$ for the extract treated groups and $p\leq0.0001$ for the chloroquine treatment arm *versus* control. The middle doses of the two extracts also lengthen the animals' life by a significant number of days at $p\leq0.05$ (AD400, 16.8 ± 0.86 days; PC400, 14.4 ± 1.07 days).

Table 1. Four-day suppressive test result of the hydroalcoholic extract of *Adhatoda schimperiana* and *Piper capense*.

Group	Parasitemia (%)	Chemosuppression (%)
NG	44.4 ± 2.73	00
AD200	36.4 ± 2.54	18.1
AD400	24.8 ± 1.56	44.1*
AD600	20.6 ± 1.47	53.6*
PC200	40.4 ± 1.91	9
PC400	30.8 ± 1.11	30.6*
PC600	22.8 ± 1.74	48.6*
CQ	1.2 ± 0.58	97.3***

The values are written as percentage mean ± SEM of parasitized red blood cell of six mice. The chemosuppression values were calculated from the NG reference point. The data is considered significant at $p\leq0.05$ as compared to the NG. The extent of significance is represented with different number of asterisks (*) and p. *$p\leq0.05$,**$p\leq0.01$, and *** $p\leq0.0001$ stands for the significant mean difference between the treatment groups and the NG at $p\leq0.05$, $p\leq0.01$ and $p\leq0.0001$, respectively. The extracts of each experimental plant were administered to three different groups at doses of 200 (group 1), 400 (group 2) and 600 mg/kg (group 3). Each group is represented as plant's abbreviated scientific name with the dose administered.

NG: Negative control (water 10 mL/kg b.w.); AD: *Adhatoda schimperiana*; PC: *Piper capense*; CQ: Chloroquine (10 mg/kg).

Table 2. Mean Survival Time of the mice treated with different doses of the hydroalcoholic extract of *Adhatoda schimperiana* and *Piper capense*.

Group	Mean Survival Time (days)
NG	8.2 ± 0.66
AD200	10.2 ± 0.86
AD400	16.8 ± 0.86*
AD600	19.6 ± 1.10*
PC200	9.0 ± 0.71
PC400	14.4 ± 1.07*
PC600	18.0 ± 1.0*
CQ	27.8 ± 0.5***

The values are written as mean ± SEM survival days of six mice. The data is considered significant at p≤0.05 as compared to the NG. The extent of significance is represented with different numbers of asterisks (*) and p. *p≤0.05,**p≤0.01, and *** p≤0.0001 stands for significant survival mean the difference between the treatment groups and the NG at p≤0.05, p≤0.01 and p≤0.0001, respectively. The extracts of each experimental plant were administered to three different groups at doses of 200 (group 1), 400 (group 2) and 600 mg/kg (group 3). Each group is represented as plant's abbreviated scientific name with the dose administered.

NG: Negative control (water 10 mL/kg b.w.); AD: *Adhatoda schimperiana*; PC: *Piper capense*; CQ: Chloroquine (10 mg/kg).

Activity on established infection (curative test)

As shown in Table 3, in the curative test was found that the extract and standard drug-treated groups progressively decrease the level of parasitemia as compared with the negative control. The level of parasitemia in the AD200, PC200, and PC400 treated mice was not significantly lower than the negative control parasitemia level throughout the days. The AD400 treated mice had significant parasitemia level lower than the negative control from the 6th day onwards at p≤0.05 on the 6th day and p≤0.01 on the 7th day. The PC600 administered mice showed significant reduction of parasitemia lately (on the 7th day, p≤0.01).

Acute toxicity test

The animals were healthy with no signs of toxicity at the maximum limit dose of 2 g/kg for each of plant extracts when observed for 14 days as per the protocol.

DISCUSSION

This study evaluated the hydro-methanolic extracts from the roots of two medicinal plants, which are commonly used in Ethiopian traditional medicine for malaria management, *in vivo* against *Plasmodium berghi*. A four-day suppressive and curative tests were employed to study the antiplasmodial effects of both extracts on early and established infections, respectively. Ultimately, percent inhibition of parasitemia, chemosuppression and mean survival time were compared among the different experimental groups to measure the antimalarial activity of each plant.

The extract of *Adhatoda schimperiana* was able to demonstrate a statistically significant (p<0.05) inhibition of parasitemia under both tests. The results from the four-day suppressive test showed 44.1 and 53.1% chemosuppression when dosed at 400 and 600 mg/kg, respectively. Similarly, the findings of the curative test indicate statistically significant inhibition of parasitemia along the five days of treatment in comparison with the negative control. In addition, there was stabilization of parasitemia over the treatment period for mice receiving 400 mg/kg while there was almost a linear reduction for those treated with 600 mg/kg of the extract. Compounds are considered to possess antimalarial activity of interest if they can bring about 30% or more parasitemia inhibition (Munoz et al., 2000). As observed in our study, the extract was able to show much higher suppression, which indicates the antiplasmodial potential of the plant. *In vivo* antimalarial activity of test substances can be rated as moderate, good or very good based on their ability to cause 50% or higher suppression at 500, 250 and 100 mg/kg, respectively (Bantie et al., 2014). According to this classification, the antiplasmodial activity of the extract can be considered as moderate. In comparison with a similar study, which evaluated the aerial parts extract of *Adhatoda schimperiana* for their *in vivo* activity against *Plasmodium berghi*, the root extract was found to have far less antiplasmodial activity. The hydroalcoholic aerial extract produced 65 to 85% chemosuppression at 400 and 600mg/kg doses, which is about a third more than the extract from the root (Petros and Melaku, 2012).

Table 3. Curative Test result of the hydroalcoholic extract of *Adhatoda schimperiana* and *Piper capense*.

Group	Parasitemia				
	3rd-day	4th-day	5th-day	6th-day	7th-day
Negative	60.40 ± 1.08	61.6 ± 2.23	67.60 ± 3.12	72.4 ± 2.70	78.0 ± 2.60
AD200	59.01 ± 2.01	58.40 ± 2.66	57.80 ± 2.90	61.20 ± 3.10	68.80 ± 2.80
AD400	63.20 ± 2.51	57.80 ± 1.31	56.4 ± 2.60	54.4 ± 2.60*	49.4 ± 2.60**
AD600	57.40 ± 2.25	59.2 ± 2.26	49.00 ± 2.85*	41.20 ± 1.24*	35.20 ± 1.6***
PC200	61.40 ± 1.86	60.40 ± 1.33	59.8 ± 1.16	66.2 ± 3.10	71.4 ± 2.20
PC400	60.20 ± 2.78	58.8 ± 1.28	58.2 ± 1.20	62.0 ± 2.50	68.4 ± 1.50
PC600	57.60 ± 3.28	57.9 ± 1.6	55.80 ± 2.01	60.40 ± 3.91	55.60 ± 2.82**
CQ	60.20 ± 2.22	49.0 ± 2.74*	17.60 ± 2.5***	4.40 ± 1.54***	0.80 ± 0.37***

The values are written as mean ± SEM of parasitized red blood cell of six mice. The data is considered significant at p≤0.05 as compared to the NG. The extent of significance is represented with different numbers of asterisks (*) and p. *p≤0.05,**p≤0.01, and *** p≤0.0001 stands for the significant mean difference between the treatment groups and the NG at p≤0.05, p≤0.01 and p≤0.0001, respectively. The extracts of each experimental plant were administered to three different groups at doses of 200 (group 1), 400 (group 2) and 600 mg/kg (group 3). Each group is represented as the plant's abbreviated scientific name with the dose administered.

NG: Negative control (water 10 mL/kg b.w.); AD: *Adhatoda schimperiana*; PC: *Piper capense*; CQ: Chloroquine (10 mg/kg).

This finding establishes the aerial parts to be of more interest for further antimalarial drug development researches. Also, it provides ground to promote the use of the aerial parts by traditional medicine practitioners should they use the plant to treat malaria in their patients.

Numerous phytochemicals have been shown to possess antimalarial action many of which belonging to alkaloids or terpenes such as iridoids, sesquiterpenes, diterpenes, terpenoid benzoquinones, steroids, quassinoids, limonoids, curcubitacins, and lanostanes. However, a number of other antimalarial compounds have also been found from flavonoids, peptides, phenylalkanoids, xanthones, and naphthopyrones (Nogueira and Lopes, 2011; Omar and Patimah, 2011). Phytochemical screening studies on the particular plant and its genus have reported on the presence of terpenoids (iridoids, diterpenoids, and triterpenoids), alkaloids, flavonoids, essential oils, lignans, vitamins, fatty acids (docosanoic acid), and salicylic acid (Bezu et al., 2015). Hence, the observed moderate antimalarial activity by the root extract *Adhatoda schimperiana* may be due to such metabolites, which have been associated with several antimalarial activities.

As evidenced by the statistically significant difference ($p<0.05$) in chemosuppression and mean survival with parasitaemia between extract treated and control groups, the hydroalcoholic root extract of *Piper capense* has also shown antimalarial efficacy augmenting both the traditional medicine claim as well as finding from a previous *in vitro* study (Chahal et al., 2011). In the four-days suppressive test, the 400 and 600 mg/kg doses of *Piper capense* have produced a statistically significant ($p<0.05$) chemosuppression of 30.6 and 48.6%, respectively. However, despite significant parasite suppression as compared to control, the middle dose (400 mg/kg) was found to be marginally active as it has only managed to show parasite inhibition almost equal to the minimum required value for extracts to be considered active i.e. parasitaemia reduction of 30% or more (Munoz et al., 2000). Though the extract managed to produce parasite inhibition to be considered active on two of the higher doses administered (400 and 600 mg/kg), the antimalarial activity is barely moderate as none of the active doses met the threshold 50% or more inhibition (Bantie et al., 2014). Similarly, a weak activity was observed in the curative test with only the 600 mg/kg dose showing a statistically significant parasitemia inhibition at the 7th day of follow-up. Both the lower and middle doses failed to significantly affect parasitemia levels in comparison with vehicle-treated mice. Therefore,

if tolerated, it may be necessary to use the *Piper capense* hydroalcoholic extract at doses greater or equal to 600 mg/kg to have better control of parasitemia on established infection. This might be due to insufficient active ingredients in the current doses and also supports the notion that all drugs cannot affect the *in vivo* experimental antimalarial model equally (Abdela et al., 2014). Generally, the observed antimalarial activity may be due to the presence of phytoconstituents belonging to phenolic compounds, quinones and primary amines, which have been shown to exhibit significant antiplasmodial activity by various studies (Kayembe et al., 2010; Thorburn, 2010; Builders et al., 2014; Bezu et al., 2015).

In addition to percent parasitemia inhibition and suppression, we determined the mean survival time for the mice used in the early infection test to further evaluate the antimalarial activity of both plants. The mean survival time was studied only in mice used in the four-day suppressive test since it is the standard *in vivo* antimalarial activity test (Kalra et al., 2006). The mean survival time is significantly higher ($p<0.05$) than the negative control group for both the extracts except for the mice administered with the 200 mg/kg of *Adhatoda schimperiana* and *Piper capense* hydroalcoholic extracts. This finding indicates the antimalarial efficacy of both plants further supplementing the evidence on parasite inhibition.

CONCLUSIONS

The hydro-methanolic root extracts of both *Adhatoda schimperiana* and *Piper capense* have demonstrated *in vivo* antimalarial activity against *Plasmodium berghi*. The extracts were able to produce statistically significant parasitemia inhibition and mean survival time increment in treated mice as compared with mice in negative control group. However, the observed antimalarial activity in the doses administered can only be rated as moderate or less for both plants. Generally, the finding of the study supports the traditional use claim and results from previous studies on the antimalarial activity of these plants. The antimalarial activities of the two plants may be due to partial or synergistic activities of the chemical constituents in the crude hydroalcoholic extracts. As a result, further phytochemical, and chronic toxicity studies should be conducted on these plants. Besides, we recommend the isolation of the constituents of the plants.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Contribution	Bobasa EM	Alemu BG	Berkessa ST	Gemechu MY	Fufa FG	Cari GZ	Rike WA
Concepts or ideas	X						X
Design	X	X					X
Definition of intellectual content	X	X	X	X	X	X	X
Literature search	X						X
Experimental studies	X						X
Data acquisition	X	X	X	X	X	X	X
Data analysis	X						X
Statistical analysis	X	X					X
Manuscript preparation	X	X	X	X	X	X	X
Manuscript editing	X	X					
Manuscript review	X	X	X	X	X	X	X

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