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IN VITRO AND *IN VIVO* ANTHELMINTIC ACTIVITY OF *FERULA COSTATA* (KOR.) AGAINST GASTROINTESTINAL NEMATODES OF SHEEP

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Abstract

This paper describes the *in vitro* anthelmintic activity of crude methanol extract (CME) and its *n*-hexane, ethyl acetate, chloroform and aqueous fractions of *Ferula costata* (Kor.), against *Haemonchus contortus* and *in vivo* activity of crude powder (CP) and CME against mixed culture of GINs. *In vitro* anthelmintic activity was determined by adult motility assay (AMA) and egg hatch test (EHT) against adult worms and eggs of *Haemonchus contortus* respectively. For *in vivo* activity, crude powder (CP) and CME of whole plant were administered to sheep infected with mixed species of GINs @ 1g, 2g & 3g kg^{-1} body weight (b.w) and the activity was estimated by reduction in eggs per gram (EPG) of faeces on days 3, 7 and 14 post treatment (PT). Based on Lethal Concentration 99% (LC_{99}) at 12 hr PT in AMA, the order of the potency of different extracts was exactly similar to the order of fractionation process of CME, i.e. CME showed the best activity (33.47 mg ml^{-1}) followed by hexane (39.77 mg ml^{-1}), ethyl acetate (42.76 mg ml^{-1}), chloroform (67.32 mg ml^{-1}) and aqueous fraction (539.27 mg ml^{-1}), while LC_{99} of positive control (Levamisole) was 1.257 mg ml^{-1}. However, differences between CME, hexane, ethyl acetate and chloroform fractions were non significant while aqueous fraction showed significantly lowest potency. The EHT showed that the activity of CME was at the top (23.08 mg ml^{-1}) and that of chloroform fraction remained at the bottom (100.32 mg ml^{-1}). However, the LC_{99} values of CME and all its fractions in EHT were non-significantly different with each other. Activities of all the extracts were significantly lower than those of positive controls both in AMA and EHT. *In vivo* administrations revealed that both CP and CME were active to variable extent. The *in vivo* anthelmintic activity increased with the increase in dose and days PT. Except the first dose of CP (1 g kg^{-1} b.w) which showed non-significant difference at day 3 and 7 PT, all the doses showed significantly different reduction in EPG compared to untreated control at all stages PT. CME @ 3g kg^{-1} exhibited the best activity on day 14 PT (47.90%) but this reduction in EPG was significantly lower than positive control (Levamisole) @7.5mg kg^{-1} b.w. (99.39%). Further *in vivo* and chemical investigations for accurate adjustment of dose and determination of active principle(s) are suggested.

Introduction

Helminthiasis has an adverse effect on production of small ruminants and hence causes heavy economic losses especially in developing countries where mismanagement and poor control practices are prevalent (Dhar *et al.,* 1982; Waller *et al.*, 1997; Githiori *et al.,* 2004; Raza *et al.*, 2007). It is a big threat to the overall industry of livestock (Saddiqi *et al*., 2010). In Pakistan, high prevalence of helminthiasis has been reported in sheep and goats (Raza *et al.,* 2007) and cattle and buffalo (Athar *et al.,* 2011). This infection is generally controlled by synthetic drugs and vaccination (Behnke *et al*., 2008). The continuously developing resistance on the part of helminths against commertial drugs has increased the interest of researchers in traditionally used medicinal plants (Wolstenholme *et al.,* 2004; Taylor *et al.,* 2009; Saeed *et al.*, 2010; Sutherland & Leathwick, 2011).

Traditional use of medicinal plants as anthelmintics has been reported from different parts of the world (Iqbal *et al.*, 2001; Lateef *et al.*, 2003; Shinwari & Gilani, 2003; Costa *et al*., 2006; Jabbar *et al.*, 2007; Qadir *et al.*, 2010; Goswami *et al.*, 2011). Anthelmintic activities of various botanical anthelmintics have been reported e.g. *Artemisia brevifolia* (Iqbal *et al.*, 2004 ; Shinwari *et al*., 2006), *Asimina triloba* (Jorge *et al.*, 2011), *Calotropis procera* (Iqbal *et al.*, 2005), *Nicotiana tabacum* (Iqbal *et al.*, 2006), *Adhatoda vasica* (Yadav & Tangpu, 2008), *Leonotis ocymifolia* (Eguale *et al.*, 2011), *Leuceana leucocephala* (Adama *et al.*, 2012), *Jatropha curcas*, *Chenopodium ambrosioides* and *Lawsonia inermis* (Eguale & Giday, 2009).

Ferula costata (Kor.) ver. Naraan (Pashto) belongs to the family Umbelliferae. It is distributed in Pakistan and Afghanistan. In Pakistan its distribution is restricted to Northern Balochistan and Khyber Pukhtoonkhwa (Nasir, 1972). Unpublished reports claim antiparasitic use *F. costata* and other species of *Ferula*. Extracts of *F. costata* (Kakar *et al.*, 2012)*, F. persica* (Shahverdi *et al*., 2005)*, F. hermonis* (Hilan *et al*., 2007) and *F. lycia* (Kose *et al*., 2010) possessed antibacterial activity. *Ferula narthex* is locally in use for gastric problems and anti-constipation (Khan *et al.*, 2011) while decoction of *Ferula assa-foetida* (fruits) showed significant activity against bacterial leaf blight (BLB) of rice (Jabeen, 2011).

Aim of the present study was to test crude methanol extract and its *n*-hexane, ethyl acetate, chloroform and aqueous fractions of *F. costata* for their *in vitro* and *in vivo* anthelmintic activities against *Haemonchus contortus* and mixed species of gastrointestinal nematodes (GINs) respectively.

Material and Methods

Plant collection and extraction: Aerial parts with mature flowers of *F. costata* were collected from Muslimbagh area of Kila Saifullah district of Balochitan-Pakistan. The plant was identified by the second author who is a plant taxonomist in botany Department University of Balochistan, Quetta. Plants were shade dried and ground to fine powder. Powdered material was kept at 4^0C till further use.

Crude Methanolic Extract (CME): CME was prepared by procedure described by Tabassam *et al.*, (2008). Powdered plant material was soaked in adequate quantity of methanol for three days at room temperature. Filtered the material and more methanol was added to it, heated to 50^0C for a while and filtered. Repeated the later procedure three times. All the filtrates were combined and dried in rotary evaporator at 45^0C. Stored the extract at 4^0C till further use.

Fractionation of CME: The CME was successively partitioned in to *n*-hexane, ethyl acetate, chloroform and aqueous fractions (Assis *et al.*, 2003). CME was suspended in an adequate quantity of 5% methanol in water. The suspension was successively partitioned with *n*-hexane, ethyl acetate, chloroform using separating funnel. All the fractions were separately dried in reduced pressure and the remaining water portion was dried as aqueous fraction.

***In vitro* anthelmintic activity:** Adult Motility Assay (AMA) and Egg Hatch Test (EHT) were adopted for determination of *in vitro* anthelmintic activity, using the procedures of Singh *et al.,* (1985) and Alawa *et al.,* (2003) respectively.

Adult motility assay: Adult *Haemonchus contortus* worms were used as test organism in AMA. The worms were collected from abomasums of freshly slaughtered sheep. Ten worms were exposed to each of the following serial dilutions of CME, its fractions and standard drug (levamisole).

i.	CME:	100, 50, 25, 12.5 & 3.13 mg ml^{-1}
ii.	Hexane fraction:	100, 50, 25, 12.5 & 3.13 mg ml^{-1}
iii.	Ethyl acetate fraction:	150, 75, 37.5, 18.75 & 9.38 mg ml^{-1}
iv.	Chloroform fraction:	150, 75, 37.5, 18.75 & 9.38 mg ml^{-1}
v.	Water fraction:	250, 125, 62.5, 31.25 & 15.63 mg ml^{-1}
vi.	Levamisole (positive control):	0.55, 0.275, 0.138, 0.069 & 0.034 mg ml^{-1}

Dilutions were made in Phosphate Buffer Saline (PBS) which was also used as negative control. Each treatment and control was used in triplicate. Worms of each treatment and control were observed for their motility after each two hours post treatment till twelve hours.

Egg hatch test: Eggs of *H. contortus* were obtained by crushing adult female worms in PBS with pestle and mortar. Debris of the mixture was removed by passing it through sieve # 400. Following treatments of CME, its fractions and positive control (oxfendazole) were made in PBS:

1.	CME and its fractions[1]:	5000, 2500, 1250, 625 & 312.5 µg ml^{-1}
2.	Oxfendazole (positive control):	3, 1.5, 0.75, 0.375 & 0.188 µg ml^{-1}
	PBS was used as negative control.	

Micro-titration plates with 24 wells were used for EHT. Two ml of each treatment and control were put in the wells in triplicates along with 250 eggs of *H. contortus*. The plates were incubated for 48 hours at 27^0C. Number of un-hatched eggs and larvae were counted in each well using inverted microscope.

***In vivo* anthelmintic activity:** The *in vivo* trails were performed at a private sheep and goat farm in the vicinity of Quetta. About one year old 24 sheep of 20-24 kg body weight (b.w.) were selected, which were naturally infected with mix species of GINs. *In vivo* activity of crude powder (CP) and CME was evaluated by Fecal Egg Count Reduction Test (FECRT) using McMaster floating procedure (Soulsby, 1982) for the count of eggs per gram (EPG) in faeces. EPG of faeces samples of each selected sheep was counted before treatment and recorded as day 0 of treatment. The selected sheep were divided into 8 groups (n=3) and treated orally with following doses:

Group 1, 2 & 3: *F. costata* CP @ 1, 2 & 3 g kg^{-1} b.w. respectively
Group 4, 5 & 6: *F. costata* CME @ 1, 2 & 3 g kg^{-1} b.w. respectively
Group 7: Levamisole HCl @ 7.5 mg kg^{-1} b.w. (positive control).
Group 8: Untreated (negative control)

Post treatment (PT) count of EPG was conducted at day 3, 7 & 14. The following formula was used for calculation of percent FECR:

$$\text{FECR (\%)} = \frac{\text{Pre Treatment EPG - Post Treatment EPG}}{\text{Pre Treatment EPG}} \times 100$$

Statistical analyses: Probit analysis (probit transformation of percentage mortality and natural logarithm transformation of dose) of data of AMA and EHT was done by statistical software "PoloPlus" (LeOra Software, 2002). Data of *in vivo* trails were subjected to one way ANOVA and LSD using computer package "SPSS".

[1]Hexane, ethyl acetate, chloroform and aqueous fractions

Results and Discussion

***In vitro* anthelmintic activity**

Adult motility assay (AMA): Table 1 shows the result of probit analysis for AMA of CME and all its fractions while Figs. 1-5 show the time and dose dependent effect

of CME, *n*-hexane, ethyl acetate, chloroform and aqueous fractions respectively. The LC_{99} indicate that CME was the most potent against adult *H. contortus* (33.47 mg ml^{-1}) followed by hexane fraction (39.77 mg ml^{-1}), ethyl acetate fraction (42.76 mg ml^{-1}), chloroform fraction (67.32 mg ml^{-1}) and aqueous fraction (539.27 mg ml^{-1}). The order of activity matches with the successive fractionation of CME. However, the overlapping 95% confidence limit (CL) showed that the above mentioned differences in results was statistically non-significant ($p>0.05$) between CME, hexane, ethyl acetate, chloroform fractions while aqueous fraction remain significantly less potent than all. The difference between LCs_{99} of extract and standard anthelmintic drug levamisole (1.257 mg ml^{-1}) was highly significant. The narrow range of 95% CL of LC_{50} is considered as good fit to log probit model.

Table 1. Data of AMA of CME of *F. costata* and its fractions and Levamisole showing LC_{50}, LC_{90} and LC_{99} (95% CL) estimates (mg ml^{-1}**) against adult worms of *Haemonchus contortus* at 12th hr PT.**

Name of extract	Slope (SE)	X^2	LC_{50} (Range)	LC_{90} (Range)	LC_{99} (Range)
CME	1.692 (±0.094)	62.03	1.41 (1.06-1.78)	8.07 (5.64-14.20)	33.47 (17.98-94.53)
Hexane fraction	1.748 (±0.092)	61.69	1.85 (1.45-2.32)	10.04 (6.98-17.61)	39.77 (21.62-106.88)
Ethyl acetate fraction	1.719 (±0.097)	62.16	1.89 (1.40-2.40)	10.55 (7.49-18.06)	42.76 (23.44-117.25)
Chloroform fraction	1.615 (±0.091)	49.79	2.44 (1.90-3.02)	15.17 (10.63-26.10)	67.32 (36.38-179.34)
Aqueous fraction	1.022 (±0.079)	34.01	2.85 (1.88-3.83)	51.17 (30.26-122.48)	539.27 (198.62-3024.54)
Levamisole (+ve control)	1.570 (±0.102)	50.520	0.042 (0.027-0.055)	0.272 (0.199-0.442)	1.257 (0.691-3.508)

Table 2. Data of EHT of CME of *F. costata* and its fractions and Oxfendazole showing LC_{50}, LC_{90} and LC_{99} (95% CL) estimates (mg ml^{-1}**) against eggs of *Haemonchus contortus* after 48 hrs incubation.**

Name of extract	Slope (SE)	X^2	LC_{50} (Range)	LC_{90} (Range)	LC_{99} (Range)
CME	1.075 (0.081)	249.3	0.73 (0.43-1.12)	4.79 (2.54-10.63)	23.08 (15.34-76.26)
Hexane fraction	1.403 (0.094)	42.2	1.01 (0.65-1.39)	8.28 (6.12-12.47)	45.97 (26.40-108.04)
Ethyl acetate fraction	1.335 (0.094)	264.1	1.28 (0.21-2.57)	11.69 (5.551-104.91)	70.90 (19.83-8678.29)
Chloroform fraction	1.494 (0.129)	171.7	2.78 (1.22-4.67)	20.04 (9.42-287.00)	100.32 (27.26-15094.45)
Aqueous fraction	2.354 (0.176)	30.0	3.01 (2.62-3.42)	10.55 (8.34-14.95)	29.33 (19.58-54.45)
Levamisole (+ve control)	2.073 (0.189)	54.070	0.0003 (0.0001-0.0005)	0.0014 (0.0010-0.0018)	0.0043 (0.0030-0.0090)

Egg Hatch Test (EHT): As shown in Table 2, the order of potency in EHT based on LC_{99} was slightly different from that of AMA. CME (23.08) was followed by aqueous fraction (29.33 mg ml^{-1}), hexane fraction (45.97 mg ml^{-1}), ethyl acetate fraction (70.90 mg ml^{-1}) and chloroform fraction (100.32 mg ml^{-1}). The overlapping rang of 95% CL of LC_{99} indicated that differences between CME and its four fractions were non-significant at $p>0.05$. As compared to extracts the positive control (oxfendazole) showed significantly high potency (4.3 µg ml^{-1}). A good fit probit model was indicated by narrow range of 95% CL of LC_{50}.

***In vivo* anthlmintic activity:** Crude powder (CP) and crude methanolic extract (CME) of *F. costata* exhibited time and dose dependent *in vivo* anthelmintic activity against mixed culture of GINs (Table 3). Both the administrations showed maximum activity at highest dose (3 g kg^{-1} b.w.) on day 14PT (Fig. 6). Like that of *Azadirachta indica* (Iqbal *et al.*, 2010) the CME in the present study also exhibited better activity than CP. Maximum reduction in EPG detected for CP @ 3g kg^{-1} b.w. at day 14 was 30.71% while at same dose and day PT it was noted for CME as 47.90%. Though both the oral administrations considerably decreased the egg counts in faeces but this activity was not comparable with that of positive control levamisole @ 7.5 mg kg^{-1} b.w. (99.39% reduction in EPG).

No plant extract of any species of *Ferula* has been evaluated for anthelmintic activity however, ferulic acid and umbelliferone isolated from *F. asafetida* have shown good *in vitro* and *in vivo* activity against larvae of *Fasciola gigantica* (Sunita & Singh, 2011). Antibacterial activities of some species of *Ferula* have also been reported (Shahverdi *et al*., 2005; Hilan *et al*., 2007; Kose *et al*., 2010; Kakar *et al.*, 2012;). In this study the CME, its *n*-hexane, ethyl acetate, chloroform and aqueous fractions and CP have shown moderate anthelmintic activity both in the *in vitro* and *in vivo* tests.

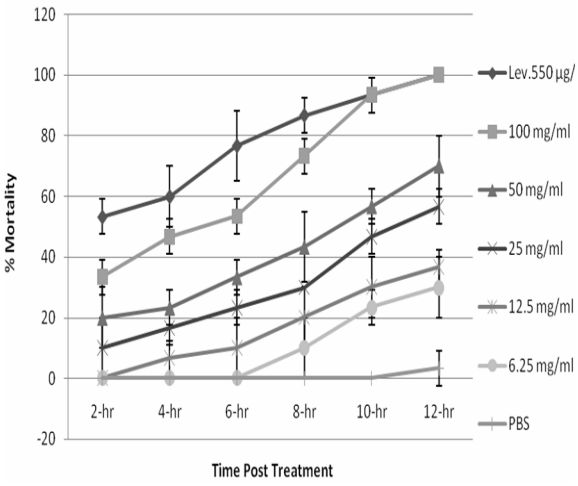

Fig. 1. Time and dose dependant response of CME of *Ferula costata* against *Haemoncus contortus* in AMA.

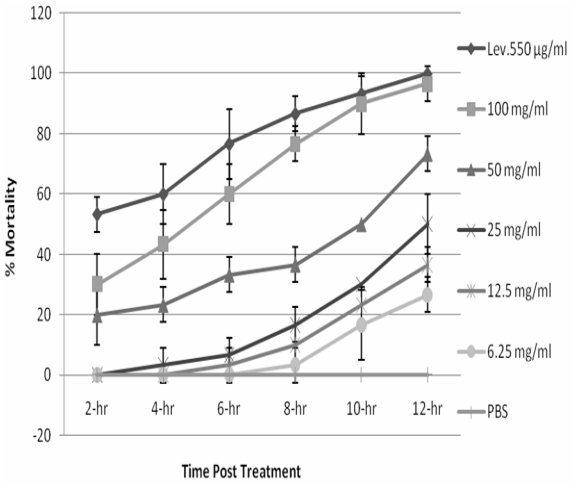

Fig. 2. Time and dose dependant response of *n*-hexane fraction of CME of *Ferula costata* against *Haemoncus contortus* in AMA.

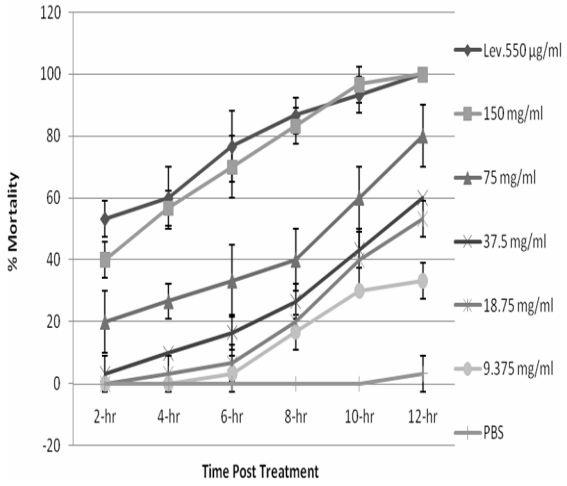

Fig. 3. Time and dose dependant response of ethyl acetate fraction of CME of *Ferula costata* against *Haemoncus contortus* in AMA.

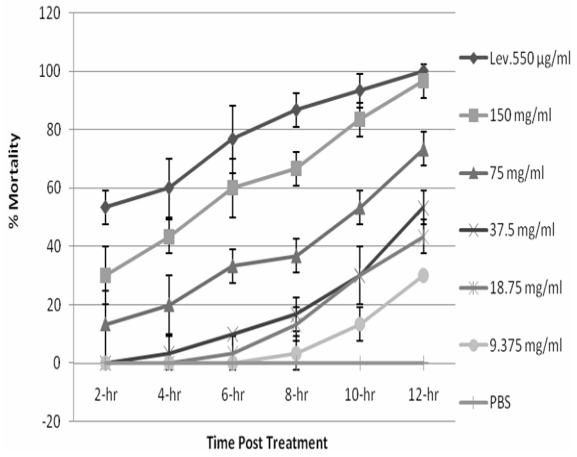

Fig. 4. Time and dose dependant response of chloroform fraction of CME of *Ferula costata* against *Haemoncus contortus* in AMA.

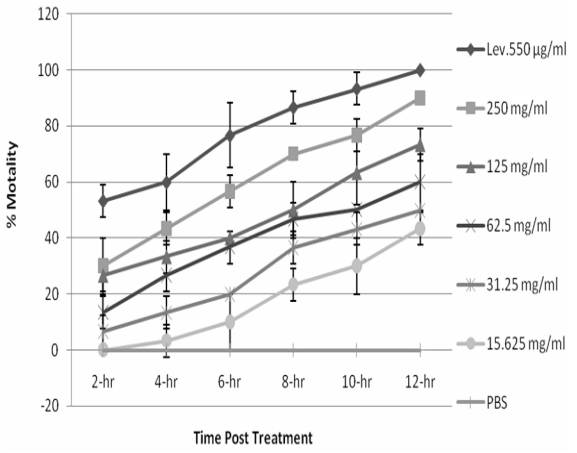

Fig. 5. Time and dose dependant response of aqueous fraction of CME of *Ferula costata* against *Haemoncus contortus* in AMA.

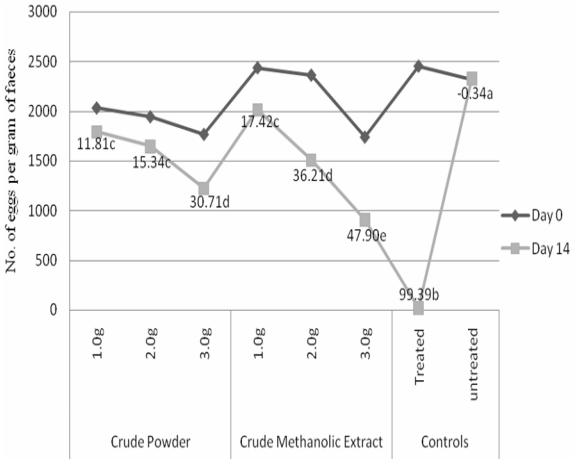

Fig. 6. Reduction in eggs per gram (EPG) of faeces in sheep treated with different doses of CP and CME of *Ferula costata* compared with controls (treated with Levamisole HCl @ 7.5 mg kg^{-1} b.w. and untreated). figures show % reduction and letters with the figures show the level of significance at $p \leq 0.05$.

Table 3. Effect of *Ferula costata* administrations on Eggs Per Gram (EPG) of faeces in sheep naturally infected with mixed species of gastrointestinal nematodes. Figures against ± show standard deviation and in () show % reduction with respect to day 0. Alphabets against () show level of significance at $p>0.05$ in a row.

Day PT*	Crude powder			Crude methanolic extract			Control	
	1.0 g	2.0 g	3.0 g	1.0 g	2.0 g	3.0 g	Treated**	Untreated
0	2032 ± 188	1943 ± 213	1765 ± 207	2439 ± 277	2364 ± 259	1737 ± 162	2455 ± 138	2323 ± 121
3	1985 ± 170 (2.31)ac	1795 ± 216 (7.62)c	1595 ± 135 (9.63)c	2240 ± 234 (8.16)c	1832 ± 208 (22.5)d	1243 ± 86 (28.44)d	22 ± 6 (99.1)b	2359 ± 147 (-1.55)a
7	1855 ± 128 (8.71)ac	1715 ± 179 (11.73)c	1355 ± 128 (23.23)de	2056 ± 233 (15.7)cd	1620 ± 139 (31.47)ef	1030 ± 71 (40.7)f	7 ± 7 (99.71)b	2288 ± 166 (1.51)a
14	1792 ± 130 (11.81)c	1645 ± 163 (15.34)c	1223 ± 140 (30.71)d	2014 ± 176 (17.42)c	1508 ± 170 (36.21)d	905 ± 65 (47.90)e	15 ± 13 (99.39)b	2331 ± 267 (-0.34)a

*; Post treatment, **; treated with Levamisole HCl @ 7.5 mg kg^{-1} b.w.

Chloroform, ethyl acetate, n-buatnol and aqueous fractions of CME of *Pterospermum acerifolium* (Parida *et al*., 2010), *Viola betonicifolia* (Muhammad *et al.*, 2012) and chloroform and ethyl acetate fractions of CME of *Ixora coccinea* roots (Surana *et al*., 2011) were found to possess anthelmintic activities to variable extent. The *n*-hexane, chloroform, ethyl acetate, butanol and aqueous fractions of *Myrsine africana* have observed antimicrobial activity (Ahmad *et al*., 2011).

Results of the current study recommend the use of CME and CP as alternative of the conventional drugs. However, further *in vivo* trails are needed for dose accuracy. Furthermore, various fractions of CME could be used for determination of active principle(s).

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