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ANALGESIC EFFECTS AND HPLC FINGERPRINTING OF *CLEOME AFRICANA* BOTSCH. EXTRACTS

Mohammed F. Abd El-Wahab[1], Mahmoud M. E. Mudawi[2], Nudrat Fatima[1] and Abdullah Naif Alshammari[3]

[1]*Department of Natural Product and Evidence based Herbal Medicine[1], College of Pharmacy, Northern Border University, Saudi Arabia.*
[2]*Department of Pharmacology, College of Pharmacy, Northern Border University, Saudi Arabia.*
[3]*Department of Clinical Pharmacy, College of Pharmacy, Northern Border University, Saudi Arabia.*
Corresponding Author: drnudrat_ekq@yahoo.com

ABSTRACT

Some plants used in Folk medicine are analgesic. Saudi Arabia is rich in medicinal plants. *Cleome africana* Botsch. is annual growing herb and has long been used medicinally for its analgesic properties among Rafha residents. The study was designed to scientifically prove its traditional use as an analgesic. For this initial phytochemical screening tests were performed with crude extract. The investigation of the peripherally acting analgesic activity of the *Cleome africana* crude extract and its ethanol, hexane, chloroform, ethyl acetate and *n*-butanol fractions was carried out in mice using Eddy's Hot plate method. The test drug exhibited significant analgesic activity by increasing the reaction time compared to control. The best result was achieved by *n*-butanol fraction of Cleome extract. To develop a standard method for identification and authentication of *Cleome africana* plant, HPLC fingerprints of crude extract was succeeded by using a Column: Symmetry C18 (5µm, 4.6*250mm) and mobile phase of Solvent A- Methanol or Acetonitrile and Solvent B-Water-formic acid (0.2%). HPLC fingerprints of different fractions were also carried out to find the pharmacological active compound. Further studies are in progress to isolate and evaluate the compounds separated by HPLC technique.

Key Words: *Cleome africana*, phytochemical screening, analgesic, Hot plate method, HPLC fingerprints

INTRODUCTION

Drug originated from plants are gaining popularity nowadays due to their resourcefulness. Medicinal Plants still are the lead role player in traditional and modern system of medicine; they provide precursors of some pharmaceuticals and neutraceuticals. Study of phytoconstituents lead to the discovery of some synthetic drugs, they verify the use of these plants traditionally as medicine and provide pathway for screening of these plants for use in some diseases of modern time (Soumya *et al*., 2009). Plants grown in Kingdom of Saudi Arabia are used traditionally to treat various ailments (Chaudhary, 2001; Ruber *et al.*, 2000, Shinwar and Khan, 2000). Drugs originated *Cleome africana* is a native to tropics and temperate zone. Its traditional use as analgesic, anti-inflammatory, antipyretic, antioxidant and antiarthritic is reported in literature (Lakshmi and Bindu, 2011).

Analgesic is a term derived from two Greek words '*an'* and '*algos'* meaning "without" and "pain" respectively (Harper, 2001). Analgesics are used to achieve relief from pain and thus called pain killers by layman. There are a number of drugs included in this category some are anesthetics which play their role by acting on Central or peripheral nervous systems blocking the sensation. Others are non-steroidal anti-inflammatory drugs comprising of salicylates and opioid drugs and the most common one is paracetamol which acts by blocking cyclo-oxygenase pathway (Dworkin *et al*, 2003)

The determination of the chromatograms for chemical compounds which are commonly found in medicinal plant extracts by High Performance Liquid Chromatography (HPLC) uses different mobile phases. These HPLC fingerprints of extracts could be used as benchmarks for the purpose of comparison when performing the qualitative and quantitative analysis.

MATERIALS AND METHODS

Plant material

The plant material was collected from Rafha city of Northern Border province, Saudi Arabia and identified by the Department of Pharmacognosy.

Qualitative analysis of secondary phytochemicals

The chemical tests including *Liebermann Burchardt test* for sterols and triterpenes, *Foam test* for saponins, *Molisch's test* for Carbohydrates and glycosides, *Mayer's* and *Dragendorff's tests* for Alkaloids, *Potassium hydroxide test* for flavonoids and *Ferric chloride test* for tannins were performed on the alcoholic extract of *Cleome africana* using the methods outlined by Harbourne, (2005) and Trease and Evans (2002).

Preparation of plant extracts

400g of the *Cleome africana* were exhaustively extracted at room temperature with 80% ethyl alcohol which yielded 85 g to dryness. The concentrated alcoholic extract was fractionated with hexane, methylene chloride, ethyl acetate, *n*- butanol and distilled water yielding 15, 13, 10 and 25 g, respectively.

Animals:

Mice, each weighting 20 - 22 g were used. The experimental mice of male sex, same age, species, strain, and weight close were chosen, kept under the same condition and taking the same food. These precautions only diminish but not completely exclude animals' variations. The animals were separated in cages comprising of 6 in each group and were maintained at room temperature. They were fed on standard chow, and allowed freely to drink water. Animals were acclimatized for one week prior to use.

Hot Plate Method:

Mice paw are highly sensitive regarding heat stimuli and they response quickly by withdrawing or licking their paw. This property is used by researches to calculate analgesic effect of centrally acting analgesics, while peripheral analgesics cannot be evaluated by this method (Vogel, 2008). Groups of 6 mice weighing between 20-22 g were used for the experiment. UGO Basile hot plate consisted of a metal surface which can be heated electrically. The temperature was adjusted to 55 ± 0.1 °C and mice were placed individually on metal surface, recording the time with stopwatch until licking / jumping starts. The time intervals used were 0, 20, 60 and 90 minutes after oral administration of control, test and standard drug. To avoid mice paw damage, the animal was withdrawn after 15 seconds (Fig. 1).

Test groups: Out of 7 groups, 3 received the alcoholic extracts orally at different doses 100, 200 and 300 mg/kg body weight, the other 4 groups were given 50 mg/kg for each fraction.
The Standard (positive control) group: received Diclofenac Na (10 mg/kg, *i.p.*)
The Negative control group: received 10 mL/kg, *i.p.* of distilled water.
The statistical analysis of data was performed using one-way ANOVA on result data. Multiple Comparisons with Dunnett t-tests were also performed. The mean, standard error of means and analysis of variance (ANOVA) were calculated by computerized programs SPSS 15.

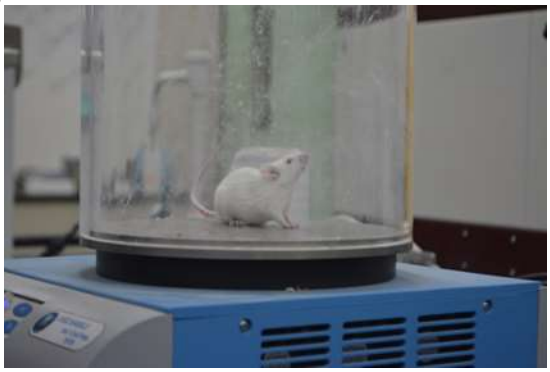

Fig. 1. Ugo Basil Hot-Plate.

HPLC fingerprints of extracts

The extracts were diluted and filter with HPLC grade methanol. The samples were analyzed using RP-HPLC, Waters® 2545 Quaternary Gradient Module pump and equipped with Waters® 2998 diode array detector, and chromatograms were recorded at 210-400 nm. The entire system was controlled using Empower 3 Software. The system has water guard Column: Symmetry C18 (5µm, 4.6*250mm) and 20 µL sample was injected by Hamilton microliter syringe. The two solvents used were Methanol as Solvent A and 2%formic acid aqueous solution as Solvent B. Detector wavelengths ranged between 210-400 nm, flow rate was adjusted at 1.4 mL/minute and program

was begun with 30% A which was held for 5 minutes, followed by 100% eluent A for the next 30 minutes and then 70% B for the next 10 minutes. The reported method was followed accordingly (Giri *et al*., 2010; Boligon *et al*., 2012).

RESULTS AND DISCUSSION

Analgesic activity:

Pain is the easiest available symptom for the diagnosis of various ailments. Apart with the treatment of disease, the patients want to get relief of pain for which many pain killers are used. But synthetic pain killers have a number of side effects and toxicities. Since 200 years ago opiates, derived from *Papaver somniferum*, are used mainly for pain management. Now most used pain killers are synthetically manufactured. In human brain there are mu, kappa and delta receptors on which these opiates act and produce analgesic, sedative and depressive effect (Harper, 2001).

Table 1. The effects of alcoholic extract and fractions of *Cleome africana* on hot plat method.

Group	(Dose mg/kg)	0 minute	30 minute	60 minute	90 minute
Ethanol 1	100	8.50±0.428	8.31±0.307	11.11±0.365	10.10±0.477
Ethanol 2	200	8.30±0.333	8.33±0.307	11.00±0.365	10.10±0.307
Ethanol 3	300	7.80±0.307	7.82±0.307	12.53±0.563	10.60±0.600
Hexane	50	7.61±0.333	7.71±0.211	10.72±0.494	10.00±0.365
Chloroform	50	6.72±0.333	7.00±0.365	8.81±0.307	9.30±0.422
Ethyl acetate	50	6.82±0.307	7.00±0.365	8.80±0.543	8.30±0.422
n-Butanol	50	6.70±0.333	6.80±0.307	12.10±0.601	11.10±0.401
Diclofenac Na	10	7.50±0.365	10.00±0.365	10.50±0.365	10.71±0.333
Distilled water	10	9.00±0.365	9.50±0.365	9.31±0.422	9.70±0.333

Data is in form of Mean Reaction Time (Seconds) ± SEM

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Table 2. ANOVA Summary.

Source	SS	df	MS	F	*P*
Treatment	54.3094	3	18.1031	16.31	≤0.0001
Error	35.517	32	1.1099		

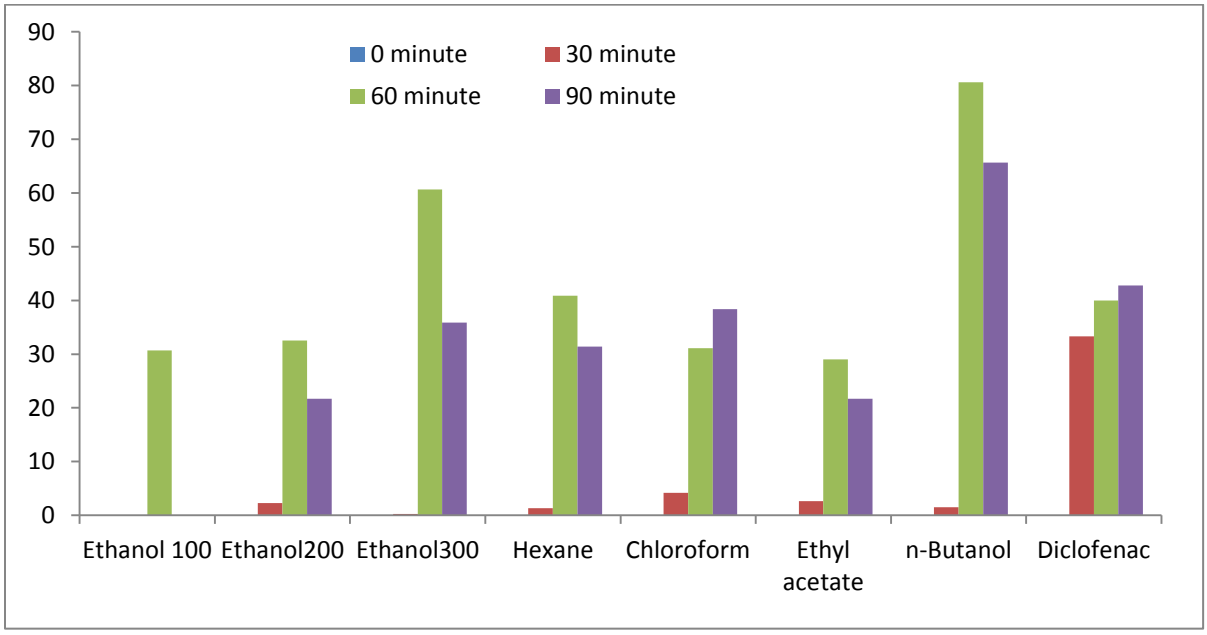

Fig. 2. Percentage inhibition of pain by crude extract and fractions of *Cleome africana*.

There is significance difference between control, standard and test groups for *Cleome africana*. The animals started jumping at 8.50 ± 0.42 seconds (100mg/kg dose) and 8.30 ± 0.33 seconds (200 mg/kg dose) at zero minutes, 8.31 ± 0.31 seconds (100mg/kg dose) and 8.33 ± 0.31 seconds (200 mg/kg dose) after 30 minutes, 11.11 ± 0.37 seconds (100mg/kg dose) and 11.00 ± 0.37 seconds (200 mg/kg dose) after 60 minutes and 10.10 ± 0.48 seconds (100mg/kg dose) and 10.10 ± 0.31 seconds (200mg/kg dose) after 90 minutes.

While 300 mg/kg dose of alcoholic extract of exerted retention time as 7.80 ± 0.31 seconds as initial, 7.82 ± 0.31 seconds after 30 minutes, 12.53 ± 0.56 seconds after 60 minutes and 10.60 ± 0.60 seconds after 90 minutes. Table 1 show that all the three doses produce analgesic effect. There is significance difference if we look at control group i.e. 9.00 ± 0.37 seconds as initial, 9.50 ± 0.37 seconds after 30 minutes, 9.31 ± 0.42 seconds after 60 minutes and 9.70 ± 0.33 after 90 minutes. The standard group retention time of mice paw was 7.50 ± 0.37 seconds as initial, 10.00 ± 0.37 seconds after 30 minutes, 10.50 ± 0.37 seconds after 60 minutes and 10.71 ± 0.33 seconds after 90 minutes. In case of Hexane fraction (50mg/kg dose) the result was 7.61 ± 0.33 seconds as initial, 7.71 ± 0.21 seconds after 30 minutes, 10.72 ± 0.49 seconds after 60 minutes and 10.00 ± 0.37 seconds after 90 minutes, chloroform fraction (50mg/kg dose) it was 6.72 ± 0.33 seconds as initial, 7.00 ± 0.37 seconds after 30 minutes, 8.81 ± 0.31 seconds after 60 minutes and 9.30 ± 0.42 seconds after 90 minutes. Ethyl acetate fraction (50mg/kg dose) gave 6.82 ± 0.31 seconds as initial, 7.00 ± 0.37 seconds after 30 minutes, 8.80 ± 0.54 seconds after 60 minutes and 8.30 ± 0.42 seconds after 90 minutes. The best result was achieved by *n*-butanol fraction (50mg/kg dose) exhibiting retention time 6.70 ± 0.33 seconds as initial, 6.80±0.31 seconds after 30 minutes, 12.10 ± 0.60 seconds after 60 minutes and seconds 11.10 ± 0.40 after 90 minutes.

In terms of percent inhibition (Fig. 2) the control group showed 0% inhibition from 0 to 90 minutes, while the standard group exhibited 33.33% after 30 minutes, 40% after 60 minutes and 42.8% after 90 minutes. This shows that Diclofenac sodium (standard group) started response earlier than test drug but the response was less than that of test drug. Ethanol at 100mg/kg dose percent inhibition was 0% at initial and after 30 minutes, 30.7% after 60 minutes decreasing to 18.82% after 90 minutes showing late and less persistent response, ethanol at 200mg/kg dose inhibited pain 2.29% after 30 minutes, 32.53% after 60 minutes and 21.69% after 90 minutes showing very little activity, same sample at higher dose of 300mg/kg responded in a different manner i.e. 0.26%, 60.64% and 35.9% after 30, 60 and 90 minutes respectively showing mild significant activity. Hexane fraction at 50mg/kg dose inhibited pain at a percent of 1.3%, 40.86% and 31.41% after 30, 60 and 90 minutes respectively. This activity can be termed as less significant in nature. Chloroform fraction also exhibited less significant activity like that of Hexane fraction i.e. 4.17%, 31.1% and 38.39% after 30, 60 and 90 minutes respectively. Ethyl acetate fraction at 50mg/kg dose exhibited least significant activity among fractions with inhibition of 2.64% after 30 minutes, 29.03% after 60 minutes and 21.7% after 90 minutes. The best activity which can be termed as highly significant was exhibited by *n*-Butanol i.e. 1.5% after 30 minutes, 80.6% after 60 minutes and 65.67% after 90 minutes. *n*-Butanol fraction effect is a delayed but prominent and longer persisting effect.

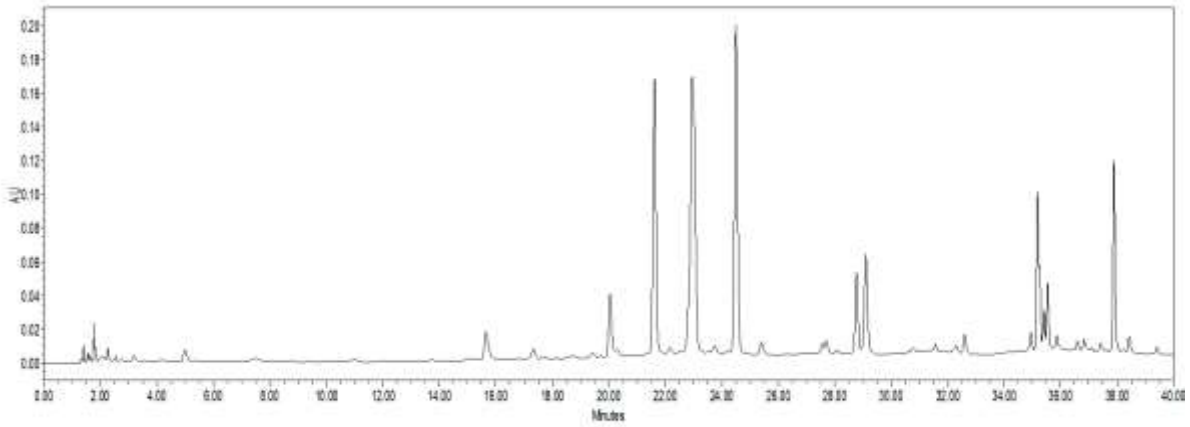

Fig. 3. HPLC Fingerprints of Crude Extract of *Cleome africana.*

HPLC fingerprints of extracts

High performance liquid chromatography has gained popularity due to its versatility, strength and range. It is very useful in assuring the quality of herbal plants. Natural products are isolated and biological assays are performed

on crude extracts. To find the active entity in this extract fractionation is carried out which make the process easier. Often the pharmacological active principle is found as minor component in crude sample for which HPLC technique plays ideal role to process rapidly such multicomponent extracts on analytical and preparative scale. Working on an isocratic system each component has a characteristic peak by the detector which can then be separated and purified (Saidharan *et al.*, 2011). HPLC fingerprint is a technique used for identification of herbal drugs and authentication of plants. This technique was performed for identification of the plant and the similar technique was used to differentiate the compounds present in various fractions of *Cleome Africana* extract.

Discussing the HPLC fingerprints, the gradient elution for crude extract was quite pronounced at different two solvent ratios. The Gradient elution for hexane and chloroform fractions was started with 30% A (held for 5 minutes) followed by 100% eluent A (for next 30 minutes) and then increasing B to 70% (for next 10 minutes). The initial peaks appeared at 1-4 minutes, 16 and 20 minutes. The major peaks in chromatogram of crude sample were obtained between 21 to 25 minutes of run, some other small peaks could be seen at 35 and 38 minutes.

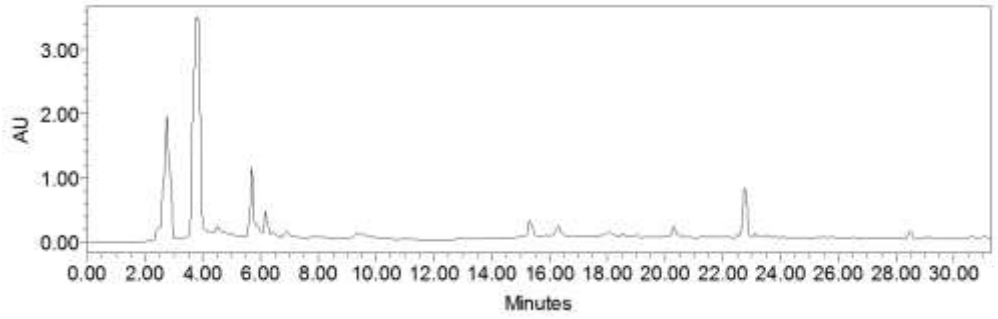

Fig. 4. HPLC Chromatogram of *Cleome africana* Hexane extract.

In Hexane extract chromatogram, the major peaks appeared at 2, 4 and 6 minutes, and small peaks were obtained at 16, 20 and 23 minutes of run.

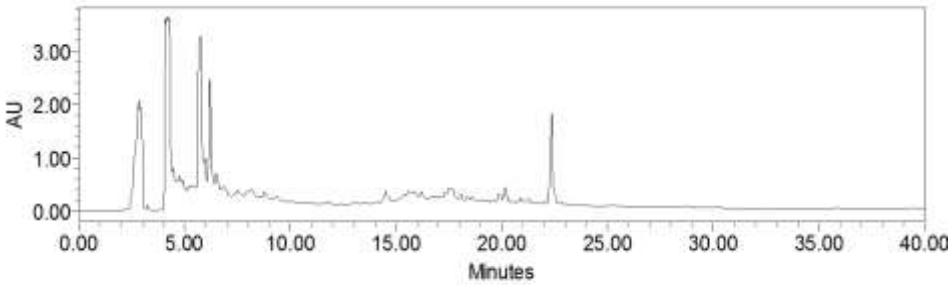

Fig. 5. HPLC Chromatogram of *Cleome africana* Chloroform extract.

In Chloroform extract chromatogram, the major peaks appeared at 3, 5 6 and 7 minutes, and small peaks were obtained between 15-20 minutes and one prominent peak appeared at 23 minutes of run.

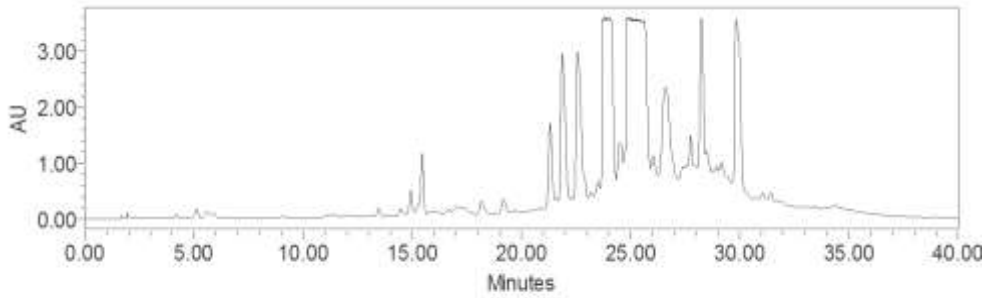

Fig. 6. HPLC Chromatogram of *Cleome africana* Ethyl acetate extract.

The Gradient elution for ethyl acetate and hexane fractions was carried out in similar pattern described above. In Ethyl acetate fraction chromatogram, the major peaks appeared continuously between 22-30 minutes and minor peaks were obtained between 15 and 16 minutes and some very small peaks appeared at 5-6 minutes of run.

In *n*-Butanol fraction chromatogram, the major peaks appeared continuously between 22-26 minutes and minor peak was obtained at 3 minute and some very small peaks appeared at 27, 28,30 and 35 minutes of run.

If we compare all chromatograms, it is observed that chromatograms of Hexane and Chloroform resemble each other and same is the case with their analgesic effect which is nearly same. Whereas in case of Ethyl acetate and *n*-Butanol, the chromatograms resemble each other but analgesic activity differs totally. For this purpose further chemical studies should be conducted in detail to explore the chemical compounds in each fraction along with crude sample which will open the doors for pharmacological screening of pure chemical compound obtained from *Cleome africana* extract.

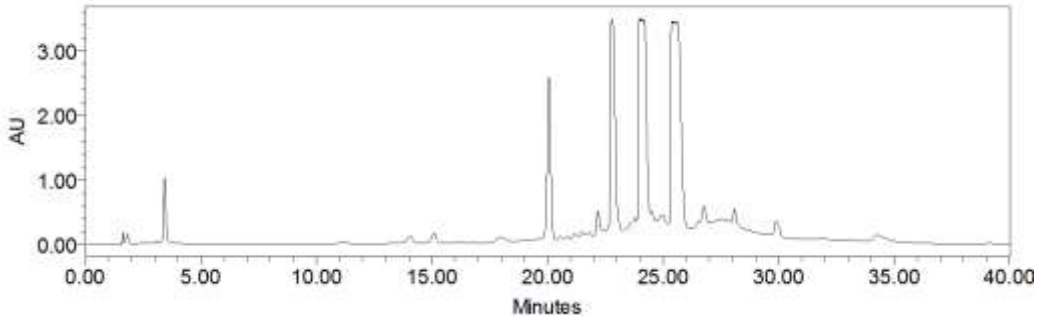

Fig. 7. HPLC Chromatogram of *Cleome africana n*-Butanol extract.

Phytochemical screening:

Phytochemical screening was carried out according to reported method which showed the presence of carbohydrates/ glycosides, saponins, flavonoids, phenolics, and steroids / triterpenes. A very little phytochemical work is found after a thorough literature search. Some researchers have reported triterpene compounds from *Cleome africana,* in which many were novel (Tsichrttzis, 1993). Others have found triterpene saponins (Masayuki, 2003). Recently some researchers have reported similar kind of triterpenes in ginseng playing role in nociception [Alessandra, 2009]. This may be possible reason of analgesic effect of *Cleome africana*.

CONCLUSION

This study revealed that *Cleome Africana* alcoholic extract produced significant dose dependent analgesic effect in mice. The best result was achieved by butanol fraction which exhibited significant analgesic effect at 50 mg/kg. Our results demonstrate that the beneficial central analgesic activities of *Cleome africana* extract supports the reported ethnomedical usage of the plant as analgesic. Further chemical studies could be conducted on crude sample and fractions of organic solvents.

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