ISOLATION AND CHARACTERIZATION OF ACTIVE CONSTITUENT OF ASHVA URINE FOR ANALGESIC ACTIVITY

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Abstract

Charak samhita has described the role of urine in anointing, pasting, enema, purgatives, fomentation and abdominal distension. Urine is used in poisoning also. Urine endowed with properties of being sharp, pungent, saline and non unctuous is useful in diseases like piles, skin disorders, white patches on skin, abdominal diseases. Urine promotes appetites and digestion. Urine acts as anti poison and kills worms in the body. Urine gives appreciable results in anemia. Ayurvedic texts have described properties of eight types of urine. Urine is used in the form of internal application by drinking and through its external application by mixing it with some powdered drugs. The objective of present work was to that horse urine an ancient medicine (Charak samhita) used for treatment of pain by tribal of India. Present proposal for research for Isolation and characterization of active constituent from Ashva urine for treatment of pain and made an effort to provide scientific claim of Ashva urine.

Keywords: Pain, analgesic activity, Ashva urine

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Introduction:

Urine-therapy has also been combined with other forms of alternative medicine. It was used by ancient Roman dentists to whiten teeth. Urine therapy has been practised for thousands of years and has merely fallen a bit into obscurity in the last century. However, urine therapy may seem to be unorthodox and perhaps revolutionary, it does not introduce anything new or original. It has been known throughout the centuries both in the West and in the East. Dr. Evagelos Danopouolos of Greece reported that urea found in urine has anti-cancerous properties. The urea seems to disrupt the ability of cancer cells to group together and kills them by upsetting some of their normal metabolic activities. Urine therapy has been used to treat cancers of the skin, cervix, lungs, eyes, breast, and liver. The first question that probably comes to mind is whether urine is not a toxic substance and how a toxic waste product could ever be of any benefit for your health. Well, urine is NOT a toxic waste product and this has been scientifically proven. 95% of urine is water, 2.5% consists of urea and the remaining 2.5% is a mixture of minerals, salt, hormones and enzymes. Toxic substances are being removed from the body through the liver and intestines, through the skin and through the outbreath. a detailed survey of the usage of different types of animal urines as medicine in the ancient Indian medical system has been attempted [1]. It may sound strange but urine's medicinal properties were discovered by our ancient sages. Charak samhita has described the role of urine in anointing, pasting, enema, purgatives, fomentation and abdominal distension. Urine is used in poisoning also. Urine endowed with properties of being sharp, pungent, saline and non unctuous is useful in diseases like piles, skin disorders, white patches on skin, abdominal diseases. Urine promotes appetites and digestion. Urine acts as anti poison and kills worms in the body. Urine gives appreciable results in anemia. Ayurvedic texts have described properties of eight types of urine. Urine is used in the form of internal application by drinking and through its external application by mixing it with some powdered drugs [2-3]. Although urine is a waste product of the body, it has multiple uses in our life. Urine contains mostly water along with few quantities of uric acid, urea, some hormones in different proportions and salts like calcium, phosphates, oxalates of sodium, etc. Many ancient practitioners of medical science have recognised the medicinal uses of urine, both externally and internally. Due to the special sanctity related to the cow in India, the cow urine is most commonly used nowadays, but the urine of other animals like elephant, goat, buffalo, camel, horse, sheep, donkey etc also have many medicinal qualities. Therefore, their urine is used for the treatment of dropsy, flatulence, worms, anaemia, abdominal enlargements, loss of appetite, poison, abdominal tumour, tuberculosis, colic, haemorrhoids, leucoderma, leprosy, amenorrhoea, and irritation of vata and kapha and in many other mental diseases. Nowadays, urine medicine is very easily available in all parts of India. It is also very good for diabetic patients. Apart from this urine is also beneficial for those suffering from jaundice. It helps in increasing the appetite and improving digestion. Urine is a watery substance produced from interstitial fluids or blood by the process of re-absorption, tubular secretion and filtration. It also serves for the homeostasis of body liquids and flushing the unwanted molecules. According to a research, it has been seen that cow urine is considered as gau jal (cow water), meaning a drink full of healthy constituents. This study supports the traditional uses of cow urine therapy. Another study claimed that distilled cow urine might help in preventing the development of kidney stones in rats [4].

In earlier ayurvedic treatises, the urine is commonly mentioned as the main ingredient in different medical recipes and therapies. Interestingly, this therapy of waste from animals was pretty much in fashion. Thus, keeping in mind the medicinal uses and properties of urine, the great sage and physicians have referred the urine as a very pungent-saline, slightly non-unctuous and sharp product, useful in poisoning, swelling of spleen, haemorrhoids, chronic skin diseases, acute distension, and fresh leprous lesions. Urine also helps in boosting up the appetite and digestion [5, 6]. Horse's urine is bitter pungent, and destroys skin diseases, wounds and neutralizes the effect of poisons. The estrogen-replacement drug Premarin, prescribed to menopausal women, is made from horse urine; in fact, the drug's name is short for PREgnant MARes' urINe. About 750,000 mares are impregnated each year for the sole purpose of collecting their estrogen-rich urine. In order to make the urine more concentrated, their water intake is restricted One of the inevitable aspects of the aging process in women is the decreased production of the hormones estrogen and progestin [7-8]. A 40% to 60% drop-off in estrogen production generally occurs with the onset of menopause (or after some types of hysterectomies). Unfortunately, for many women these changes often are accompanied by uncomfortable symptoms (such as hot flashes, night sweats, and vaginal dryness), and estrogen loss is also linked to a significant increase in the

incidence of heart disease, stroke, and osteoporosis. Premarin (and Premarin-containing products such as Prempro, Premphase, and Prempac) is a drug used in hormone replace therapy HRT regimens prescribed for women to help lessen the symptoms of menopause and reduce the risk of heart disease, stroke, and osteoporosis. Premarin is available in several forms (pills, creams, injections, patches) and, as the name (a shortening of the phrase "pregnant mares' urine") suggests, contains conjugated estrogens obtained from the urine of pregnant mares [9-10]. Analgesics are drugs that act on peripheral or central nervous system to selectively relieve pain without significantly altering consciousness. Centrally acting analgesics act by raising the threshold for pain and also altering the physiological response to pain. On the other hand, peripherally acting analgesics act by inhibiting the generation of impulses at chemoreceptor site of pain [11-12]. The animal models employed for screening of analgesic activity in this study are pain-state models using thermal stimuli which include tail-flick and hot plate methods. Both methods are useful in illustrating centrally mediated antinociceptive responses which focus generally on changes above the spinal cord level. While the tail-flick method mediates a spinal reflex to a nociceptive stimulus, hot plate method involves higher brain functions and is regarded a supraspinally organized response.

Materials and Methods

Collection of Horse Urine: Horse mare urine was collected in mid-stream using a polypropylene tube.

Qualitative estimation of urine

Preliminary identification of urine samples: The colleceted horse urine samples were examined for the physical characteristeics like color, odor, taste etc. The fresh urine sample should be examined immediately bacteria could increase glucose and urea decomposition, therefore urine becomes alkaline due to the liberation of ammonia. If a urine sample cannot be analyzed while fresh, it can be refrigerated. The typical analysis includes:

Clarity and color: Normally, urine is clear and yellowish, although turbidity may appear occasionally due to mucus or precipitated salts (urates or phosphate). Abnormal turbidity may be due to large quantities of cells (red, white or epithelial) or to bacteria. The terms of "clear", "cloudy" or "turbid" are usually appeared in the report urine analysis. The color of urine excreted by a healthy subject is due to urochrome pigments, which ranges from almost colorless, dark to yellowish. The color of urine depends mainly upon the concentration of the sample, the more concentrated specimens usually being a dark yellow. Urine is a complex aqueous solution of inorganic salts and inorganic waste products of body metabolism. An average sample of urine has: (4%) solids dissolved in (96%) water. Of these, approximately (2%) is urea, (1%) NaCl and all the other organic and inorganic constituents make up the remaining (1%). The inorganic substances in urine are urea, uric acid, creatinine and ammonium salts. Among the inorganic substances presents in urine are Na+, Cl- , HCO3- , K+, Ca+2, Mg+2 , PO4-3 and SO4-2.

Specific gravity (sp. gr.): Specific gravity depends on the number, density and weight of dissolved particles in urine. The Specific gravity of urine is variable over a (24hr.) period. Normally, the range is between (1.008-1.025). Specific gravity could be measured using a urinometer, which is a hydrometer has been adapted to measure sp. gr.

Analytical parameter identification by HPLC method: A urine samples were obtained at the routine laboratory and were declared as negative in the screening for prohibited substances. They were selected on the basis of a large range of visually accessible properties (color, viscosity and so on). The reference urine sample was centrifuged at 5000 rpm for 30 min and for every sample an aliquot (10 µl) of the supernatant was collected. The analytical samples were randomly injected into the HPLC system (Shimadzu LC1080 with PDA detectror, column C18 phenomenox).

Mobile phase: Acetonitrile: Water: Acetic acid (70:30:0.1)
Run time: 50 min.
Flow rate: 1ml/min.

Isolation of active constituents from urine sample: Urine was adjusted to pH 3-0 with conc. HCl, (NH4)2SO4 was added (50g./lOOml.) and were diluted into ether-ethanol (3:1, v/v). The diluted materials were filtered and evaporated to dryness under reduced pressure in a rotary evaporator and evaporated to dryness. The residue was dissolved in benzene (200ml.) and the solution extracted thoroughly with water. The water fractions were extracted with methylene dichloride, which was dried and evaporated to dryness. Final purification was by

chromatography on thin layers of silica gel. Dissolved in benzene, the material was divided between ten plates (each 20cm. x 20 cm.) and applied as a strip. The plates were developed in solvent system (chloroform: methanol; 9:1), and the compound was located by UV at 264 nm. Two seperates band were scraped from TLC plates and compound was recovered from silica with chloroform: ethyl acetate (1:1, v/v) filtered and oranic phase were evaporated to dryness under reduced pressure in a rotary evaporator. The procedures were repeated for all TLC plates. The TLC of isolated compound was performed showing only a single spot. Two compounds (compound I and Compound II) were isolated using preparative thin layer chtomatographic method.

Characterization of isolated compounds: Compounds (compound I and Compound II)isolated from horse urine were characterized by various methods like Uv-visible spectroscopy, FT-IR analysis, 1H-NMR,13C-NMR, Elemental Analysis, HPLC, HPTLC, MASS Spectrometry

HPTLC: HPTLC was carried out on pre-coated TLC-plates, silica gel60 F-254, 20 x 20 cm (Merck, Darmstadt, FRG). Samples were applied on to Aluminum backed pre-coated Merck silica gel plate 60 F254 plate (10×10 cm) using Camag automatic TLC sampler 4 attached to Camag HPTLC system. Approximately 3 μL each of all test solutions were loaded in the form of bands with bandwidth of 8 mm using Hamilton syringe (100 μL). The plate was developed in a solvent system, Toluene: Ethyl acetate: formic acid (7:3:0.2) using a twin trough chamber upto a distance of 9 cm. After the run, the plate was observed under UV light at 254 nm and 366 nm. The Rf value and color of the resolved bands were recorded. Densitometric scanning of the plates at 254 nm and 366 nm was done by using Camag TLC scanner 3.

Selection of animals and preparation of groups: Healthy albino rats of either sex, weighing between 180-250g and housed in standard environmental conditions of temperature, humidity, and light and provided with standard rodent food and water ad libitum. The animals were cared and used in accordance with the CPCSEA guidelines and experimental protocols approved by institutional animal ethics committee.

Acute Toxicity studies of *isolated compound* as per OECD guideline: Acute toxicity studies of isolated compound were performed in Swiss Albino female mice (25 to 30 g) dose levels of 10, 100 and 500 mg/kg as per OECD guide lines. No mortality was observed in rats dosed with the mammeigin isolated compound and phlorizin isolated compound at dose levels of 10, 100 and 500 mg/kg (p.o). The treated animals did not demonstrate any significant changes in behavioral pattern and exhibited normal activity. Also there were no clinical signs of tremors, convulsions, exophthalmos, salivation, diarrhea and lethargy. There was no significant difference in the mean body weights between treated groups and control group and the rats exhibited normal body weight gain during the study. No lethal effects or mortality was observed in animals throughout the test period following single oral administration at all selected dose levels of all mammeigin isolated compound and phlorizin isolated compound. The animals were examined for long term toxicity (14 days).

In-Vivo Analgesic Study Design: Mature albino Wistar rats (150–200 g) of both sexes were used for the experiment. The animals were housed under standard laboratory conditions at room temperature with relative humidity of 70–80%. They were fed with standard commercial diet and water *ad libitum*. Prior to the experiment, the animals were fasted for 12 h with water given *ad libitum* and weighed. The rats were randomly assigned to four groups of six animals each for the two different experimental models. The first group served as negative control receiving normal saline (10 mL/kg). The second and third groups served as positive control and were given standard drugs, morphine sulfate and sodium salicylate, respectively (10 mg/kg each). The mammeigin isolated compound and phlorizin isolated compound were given at a dose of 20 mg/kg to the group. All treatments were administered intraperitoneally.

Tail-Flick Test: Antinociceptive (analgesic) activity of the mammeigin isolated compound and phlorizin isolated compound was evaluated by the tail-flick method. About 5 cm from the distal end of the tail of each rat was immersed in warm water maintained at 50°C. The reaction time (in seconds) was the time taken by the rat to flick its tail due to pain. The first reading was omitted and reaction time was taken as the average of the next two readings. The reaction time was recorded before (0 min) and at 15, 30, 45, and 60 min after the administration of the treatments. The maximum reaction time was fixed at 15 sec to prevent any tail tissue injury. If the reading exceeds 15 sec, it would be considered as

maximum analgesia. The maximum possible analgesia (MPA) was calculated as follows:

$$\text{MPA} = \frac{\text{Reaction time for treatment} - \text{reaction time for saline}}{15\,\text{sec} - \text{reaction time for saline}} \times 100.$$

Hot Plate Test: Evaluation of analgesic activity of the mammeigin isolated compound and phlorizin isolated compound were also carried out using hot plate method. The rats were placed on a hot plate maintained at 55°C within the restrainer. The reaction time (in seconds) or latency period was determined as the time taken for the rats to react to the thermal pain by licking their paws or jumping. The reaction time was recorded before (0 min) and at 15, 30, 45, and 60 min after the administration of the treatments. The maximum reaction time was fixed at 45 sec to prevent any injury to the tissues of the paws. If the reading exceeds 45 sec, it would be considered as maximum analgesia. The maximum possible analgesia (MPA) was calculated as follows [13-14]:

$$\text{MPA} = \frac{\text{Reaction time for treatment} - \text{reaction time for saline}}{45\,\text{sec} - \text{reaction time for saline}} \times 100.$$

Result and Discussion

Qualitative estimation of urine: The horse urine is invariably turbid, due to the suspension of the carbonates of lime and magnesia which precipitate themselves in still greater abundance as the urine cools and stands, and undergoes ammoniacal fermentation. The amount of salts in suspension is in some cases remarkable, the most common being the carbonates of lime and magnesia, the majority of my analyses estimated separately as suspended lime and magnesia. Boiling the urine by driving off C 02 precipitates more of the lime salts. In one or two cases after the urine had stood some days, a hard scum, quite crystalline, has formed on its surface ; this has consisted of crystals of lime carbonate. Only once in ninety-six observations had I a perfectly clear urine presented for examination, a urine which threw down no deposit on cooling and standing, and was in most of its physical features closely allied to human urine. Fresh urine has a faint but distinctly ammoniacal sm ell; the fluid which represents the twenty-four hours' excretion is always powerfully ammoniacal. This latter creates a difficulty with regard to the determination of urea, for it is impossible to say how much of the ammonia is due to changes in the urea, and how much is preformed ammonia.

Isolation of active constituents from Horse urine (Compound 1: Mammeigin): Two compounds (compound I and Compound II) were isolated using preparative thin layer chtomatographic method.

Characterization of isolated compounds: Compounds (compound I and Compound II) isolated from horse urine were characterized by various methods like Uv-visible spectroscopy, FT-IR analysis, 1H-NMR, 13C-NMR, Elemental Analysis, HPLC
HPTLC, MASS Spectrometry

Compound 1: Mammeigin
Molecular Formula: C25H24O5
Molecular Weight: 404.5 g/mol
IUPAC name 5-hydroxy-8,8-dimethyl-6-(3-methylbutanoyl)-4-phenyl-2H,8H-pyrano[2,3-f]chromen-2-one

Isolation of active constituents from Horse urine (Compound II Phlorizin): Compound II: Phlorizin
A white solid, samples often appear yellow owing to impurities. It is of sweet taste and contains four molecules of water in the crystal.
Molecular Formula: C21H24O10
Molecular Weight: 436.4 g/mol
IUPAC Name: 1-[2,4-dihydroxy-6-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl) oxan-2-yl]oxyphenyl]-3-(4-hydroxyphenyl)propan-1-one
Melting Point: 110.0 °C

In vivo Aalgesic activity

Tail-Flick Test: The results of the analgesic activity of the Mammeigin isolated compound and Phlorizin isolated compound are shown in **Table 4-5.** Animals treated with normal saline (negative control) did not show any significant difference in the reaction time on tail-flick throughout the 60 min observation. In comparison with the baseline values within the same treatment groups, the increase in reaction time at different time points significantly differed for morphine sulfate only. Duration of the reaction time in morphine sulfate and Mammeigin isolated compound and Phlorizin isolated compound treated animals was significantly higher compared to saline treated animals, except for Mammeigin isolated compound and Phlorizin isolated compound group at 60 min. The highest reaction time for the Mammeigin isolated compound treated group was 8.02 sec at 30 min, and Phlorizin isolated compound group was 7.31 sec at 30 min. While it was 4.53 sec and 11.98 sec for

saline and morphine sulfate groups, respectively. At all time points, the tail-flick latency time differed significantly between the extract and morphine sulfate groups, being greater for the latter group. No significant difference in reaction time was observed between the chloroform extract and sodium salicylate. Observation in rats treated with sodium salicylate did not give any significant analgesic effect in comparison with baseline values, saline, or Mammeigin isolated compound and Phlorizin isolated compound (except for 30 min after treatment).

The analgesic effect of morphine sulfate was evident within 15 min following intraperitoneal administration. The maximum possible analgesia (MPA) remained elevated during the observation period, reaching its peak at 60 min (81.0%). Likewise, the methanol extract also showed analgesic activity beginning at 15 min, with the highest MPA at 30 min, and gradually decreased towards 60 min. For sodium salicylate, the maximum possible analgesia exhibited similar trend, producing a peak at the same time point (22.0%). With reference to MPA value, the methanol extract demonstrated stronger analgesic activity than sodium salicylate at all time points.

Hot Plate Test: The results of the analgesic effect of Mammeigin isolated compound and Phlorizin isolated compound using hot plate method are presented in **Table 6-7**. The results showed that there was no significant difference on the thermal stimulus in rats treated with normal saline (negative control) throughout the 60 min observation. There was no increase in reaction time at all time points compared to baseline values (0 min) within the same treatment groups. In comparison to the saline treated animals, the significant increase in the reaction time to thermal pain was not detectable in both sodium salicylate and extract with the exception of morphine sulfate. However, the observation in morphine sulfate treated animals is only noted at 45 and 60 min. The reaction time was significantly different between the extract and morphine sulfate, being greater for morphine sulfate at 30, 45, and 60 min after treatment. No significant difference was observed between the extract and sodium salicylate. Morphine sulfate elicited significant analgesic activity within 15 min following administration as evidenced by the gradual increase throughout the observation period. At the peak of activity (45 min), morphine sulfate showed maximum possible analgesia of 84.7%. Rats treated with sodium salicylate exhibited analgesic activity at a slower interval, which began at 45 min (48.1%) and then declined. On the basis of these findings, tail-flick is a better method to evaluate analgesic activity compared to hot plate as no significant results were observed for all treatments using hot plate with the exception of morphine sulfate.

In tail-flick model, the Mammeigin isolated compound and Phlorizin isolated compound exhibited significant analgesic activity by increasing the reaction time of the rats compared to control (saline treated rats) at all time points, except at 60 min. Sodium salicylate and morphine sulfate were used as reference drugs, which are considered mild and moderate to severe analgesics, respectively. In comparison with control, morphine produced the most significant antinociception effect during all observation times, followed by the Mammeigin isolated compound and Phlorizin isolated compound, while no significant analgesic effect was observed for sodium salicylate. The tail-flick method is based on the observation that morphine-like compounds are selectively able to prolong the reaction time of typical tail-withdrawal effect in rats. This method is also useful in differentiating central opioid-like analgesics from peripheral analgesics. The analgesic effect of the Mammeigin isolated compound and Phlorizin isolated compound on this pain-state model indicates that it might be centrally acting. With reference to the MPA value, the analgesic effects of Mammeigin isolated compound and Phlorizin isolated compound and morphine sulfate were evident within 15 min following intraperitoneal administration. The tail-flick latency of the Mammeigin isolated compound and Phlorizin isolated compound at all time points was less than that of reference drug, morphine sulfate, which is a slow onset opioid with long duration of action. Although there was no significant analgesic effect between the reaction time of Mammeigin isolated compound and sodium salicylate, and Phlorizin isolated compound exhibited a non-significant trend of higher reaction time compared to sodium salicylate. Both treatments produced comparable reaction times, suggesting that the Mammeigin isolated compound and Phlorizin isolated compound could be a better natural alternative for mild pain relief.

In conclusion, mammeigin isolated compound and phlorizin isolated compound displayed analgesic activity and supported the traditional use of this plant in pain relief. Further study is necessary to identify the active compounds present in this extract and to elucidate the mechanisms involved in its analgesic properties.

Summary and Conclusion: Although urine is a waste product of the body, it has multiple uses in our life. Urine contains mostly water along with few quantities of uric acid, urea, some hormones in different proportions and salts like calcium, phosphates, oxalates of sodium, etc. Many ancient practitioners of medical science have recognised the medicinal uses of urine, both externally and internally. Horse is the major source of horse urine. In Maharashtra Nandurbar district found horse urine which can use for treatment of inflammation and pain. In Horse urine, hormones, proteins, antibodies and other beneficial pharmacological agents found which are helpful in treatment of various diseases. Research for Anti-inflammatory and analgesic activity of this urine is helpful to scientific claim. Two compounds (compound I and Compound II) were isolated from horse urine using preparative thin layer chtomatographic method. Compounds (compound I and Compound II) isolated from horse urine were characterized by various methods like Uv-visible spectroscopy, FT-IR analysis, 1H-NMR, 13C-NMR, Elemental Analysis, HPLC, HPTLC, MASS Spectrometry. On the basis of literature compound I is identified as Mammeigin and compound II is charecterized as phlorizin The results of the analgesic effect of of mammeigin and phlorizin isolated compound using hot plate method. The reaction time was significantly different between the mammeigin isolated compound and phlorizin isolated compound and morphine sulfate, being greater for morphine sulfate at 30, 45, and 60 min after treatment. No significant difference was observed between the of mammeigin and phlorizin isolated compound and sodium salicylate. Morphine sulfate elicited significant analgesic activity within 15 min following administration as evidenced by the gradual increase throughout the observation period. At the peak of activity (45 min), morphine sulfate showed maximum possible analgesia of 84.7%. Rats treated with sodium salicylate exhibited analgesic activity at a slower interval, which began at 45 min (48.1%) and then declined. On the basis of these findings, tail-flick is a better method to evaluate analgesic activity compared to hot plate as no significant results were observed for all treatments using hot plate with the exception of morphine sulfate. In tail-flick model, the mammeigin and phlorizin isolated compound exhibited significant analgesic activity by increasing the reaction time of the rats compared to control (saline treated rats) at all time points, except at 60 min. Sodium salicylate and morphine sulfate were used as reference drugs, which are considered mild and moderate to severe analgesics, respectively. In comparison with control, morphine produced the most significant antinociception effect during all observation times, followed by the of mammeigin and phlorizin isolated compound, while no significant analgesic effect was observed for sodium salicylate. The tail-flick method is based on the observation that morphine-like compounds are selectively able to prolong the reaction time of typical tail-withdrawal effect in rats. This method is also useful in differentiating central opioid-like analgesics from peripheral analgesics. The analgesic effect of the mammeigin isolated compound and phlorizin isolated compound on this pain-state model indicates that it might be centrally acting. With reference to the MPA value, the analgesic effects Mammeigin isolated compound and Phlorizin isolated compound and morphine sulfate were evident within 15 min following intraperitoneal administration. The tail-flick latency of the Mammeigin isolated compound and Phlorizin isolated compound at all time points was less than that of reference drug, morphine sulfate, which is a slow onset opioid with long duration of action. Although there was no significant analgesic effect between the reaction time of the Mammeigin isolated compound and Phlorizin isolated compound exhibited a non-significant trend of higher reaction time compared to sodium salicylate. Both treatments produced comparable reaction times, suggesting that the of mammeigin and phlorizin isolated compound could be a better natural alternative for mild pain relief. In conclusion, the current research provides the scientific support for the ethno medicinal use of the mammeigin and phlorizin isolated compound studied and provides the presence of natural anti-inflammatory activity. The results also substantiate the prospective of mammeigin and phlorizin isolated compound as a source of anti-inflammatory with effective analgesic lead molecules for pharmaceutical interest. Both typers of isolated compound displayed have traditional use of for pain relief.

Table 1: Physical characterization of horse urine

Test	**Values**
Color	Straw to amber
Odour	Strong
Taste	Pungent, bitter
Specific gravity	1.036
pH	5.6 – 8.0
Total solids	230
Sugar	Nil
Ketone bodies	Nil
Protein	Nil
Creatinin	1-2 g/24h
Bile salts	Nil
Bilirubin	Nil
Urobillinogen	1-4 mg/24h

Table 2: Qualitative estimation of Organic substance in urine

Organic substance	**Concentration (mg/dl)**
Glucose	0
Urea	1820
Uric acid	50
Creatinine	190
Protein	0
Amonia	700 g daily
Benzoic acid	6.5
Hippuric acid	12.57

Table 3: Qualitative estimation of Inorganic substance in urine

Inorganic substance	**Concentration (mg/dl)**
Na+	125
K+	60
Ca-2	5
Mg+2	15
Cl-	130
HCO-3	14
SO_4^{-2}	33
PO_4^{-3}	40

Table 4: Analgesic effect of Mammeigin isolated compound by tail-flick method in rats

Treatment	**Mean reaction time (sec)**				
	0	**15**	**30**	**45**	**60**
Control	4.25	4.51	4.53	4.58	5.17
Morphine sulphate	6.50	11.21	11.98	12.91	13.23
Sodium salicylate	4.15	5.30	6.76	6.78	6.80
Mammeigin	6.64	7.02	8.02	7.03	6.69

Table 5: Analgesic effect of Phlorizin isolated compound by tail-flick method in rats

Treatment	**Mean reaction time (sec)**				
	0	**15**	**30**	**45**	**60**
Control	4.25	4.51	4.53	4.58	5.17
Morphine sulphate	6.51	11.11	11.81	12.73	13.813
Sodium salicylate	4.25	5.24	6.81	6.91	6.99
Phlorizin	6.02	6.51	7.31	6.62	6.05

Table 6: Effect of Mammeigin isolated compound by hot plate test method

Treatment	Mean reaction time (sec)				
	0	**15**	**30**	**45**	**60**
Control	29.21	24.51	26.43	23.58	21.97
Morphine sulphate	29.21	37.21	41.60	41.71	41.83
Sodium salicylate	29.21	27.30	31.76	34.36	34.80
Mammeigin	29.21	28.30	32.76	35.36	34.98

Table 7: Effect of Phlorizin isolated compound by hot plate test method

Treatment	Mean reaction time (sec)				
	0	**15**	**30**	**45**	**60**
Control	29.21	24.51	26.43	23.58	21.97
Morphine sulphate	29.21	37.21	41.60	41.71	41.83
Sodium salicylate	29.21	27.30	31.76	34.36	34.80
Phlorizin	29.21	25.09	24.12	25.93	25.29

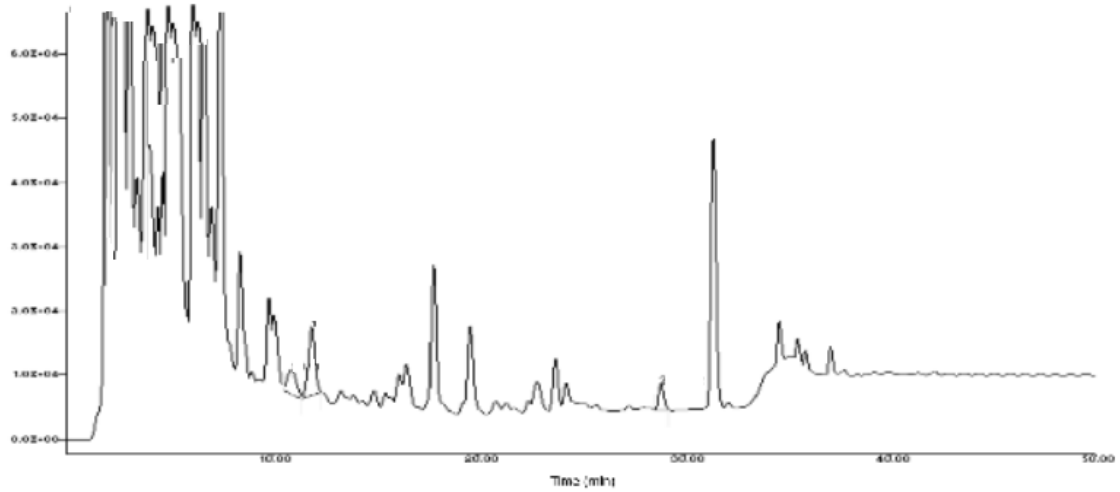


Figure 1: HPLC analysis of horse urine sample

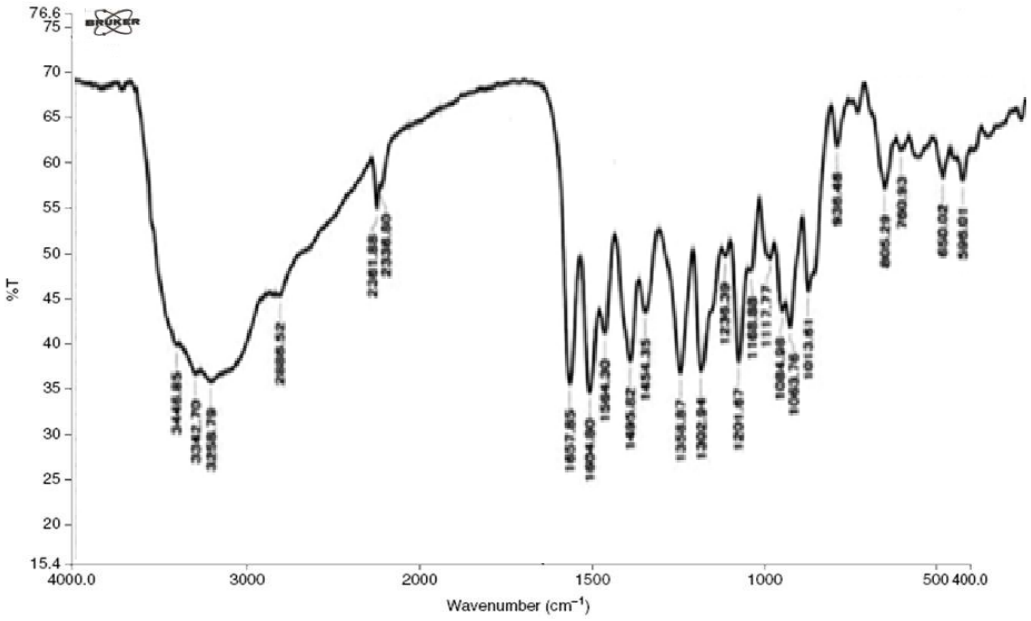


Figure 2: IR spectra of compound I

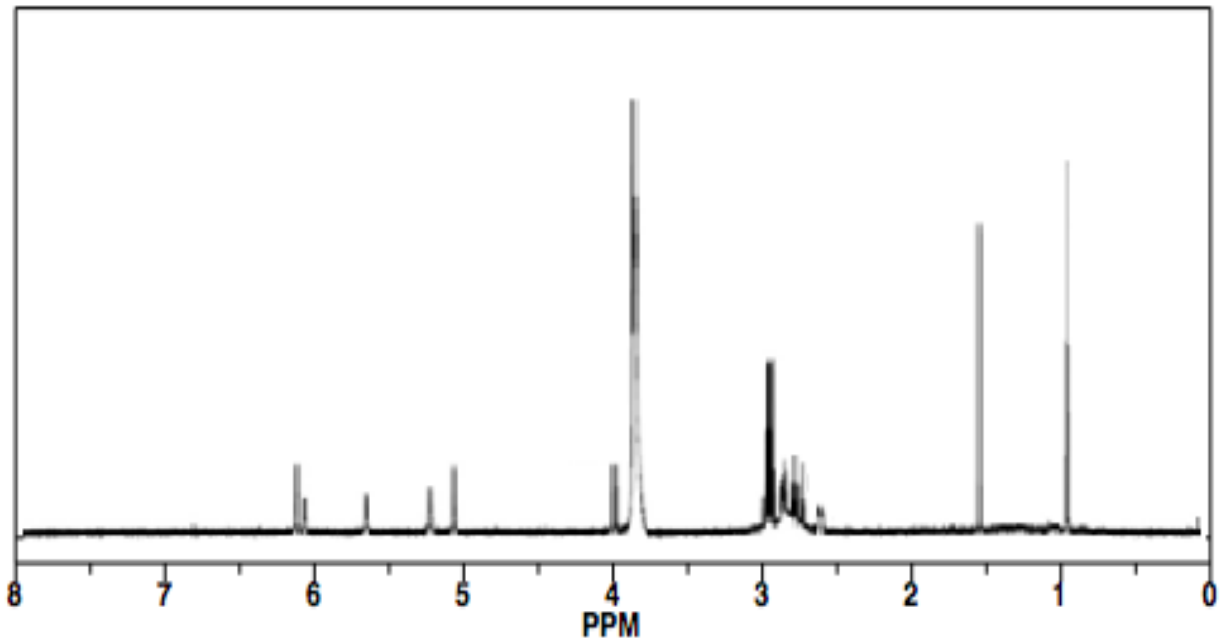


Figure 3: 1HNMR of compound I

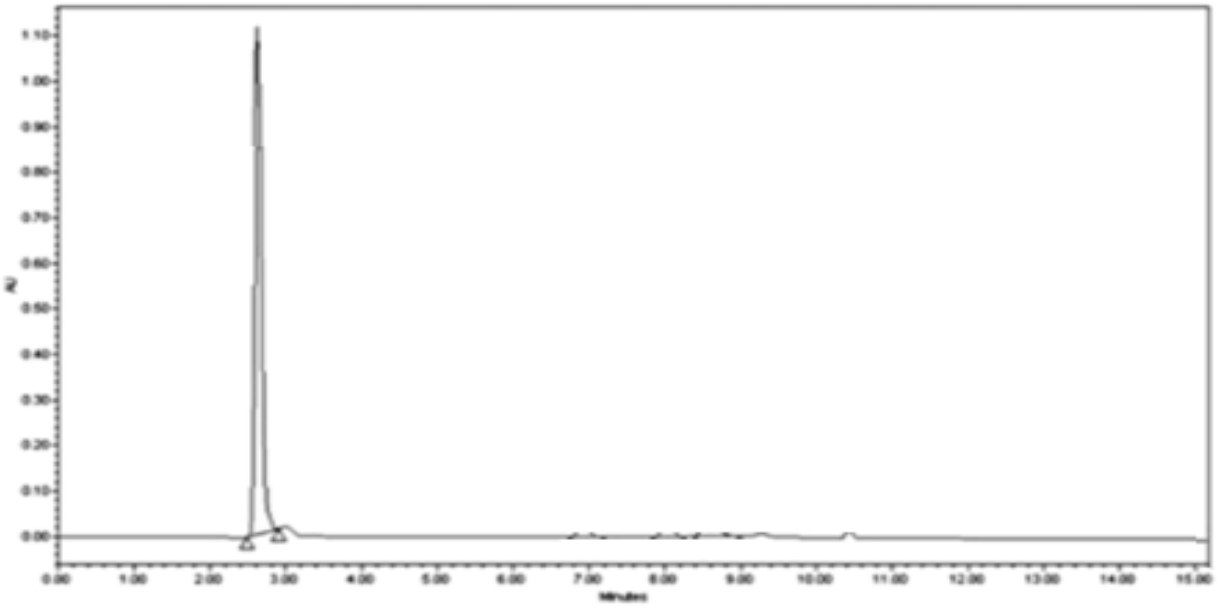


Figure 4: HPLC chromatogram of compound I

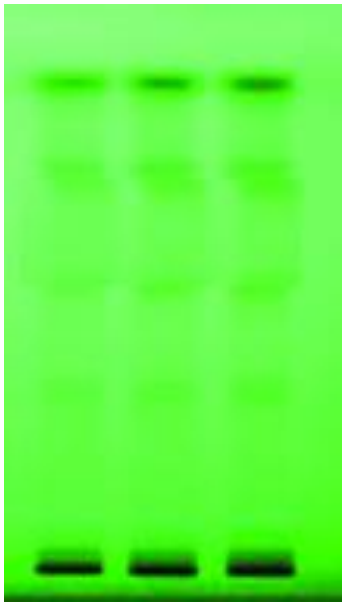


Figure 5: HPTLC of compound I

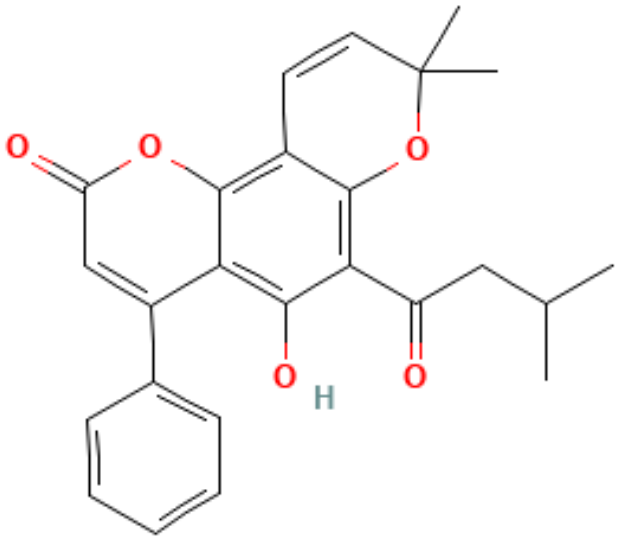


Figure 6: Structure of Compound I (Mammeigin)

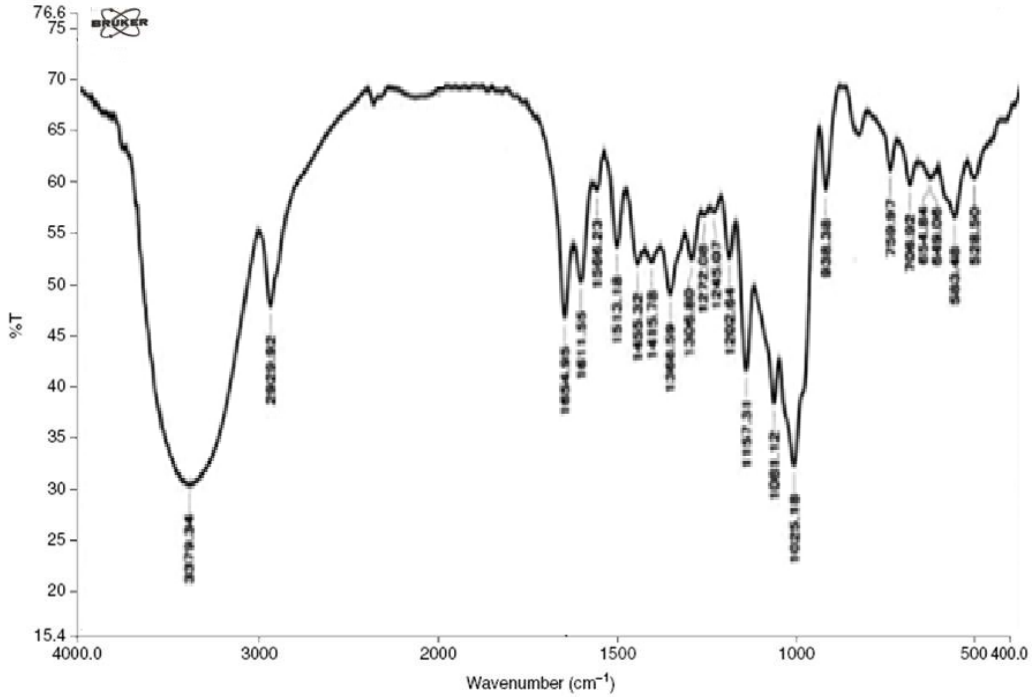


Figure 7: FTIR of Compound II

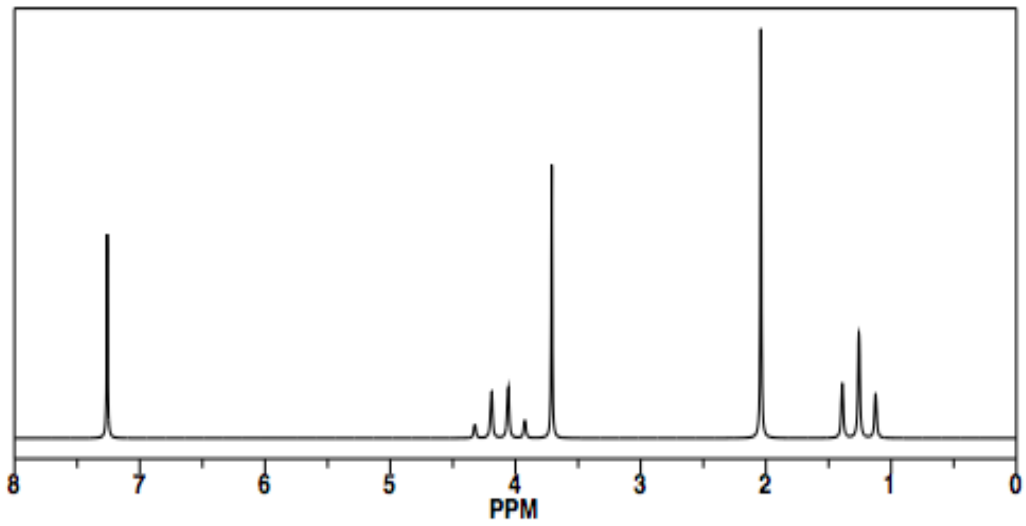


Figure 8: 1H NMR of Compound II

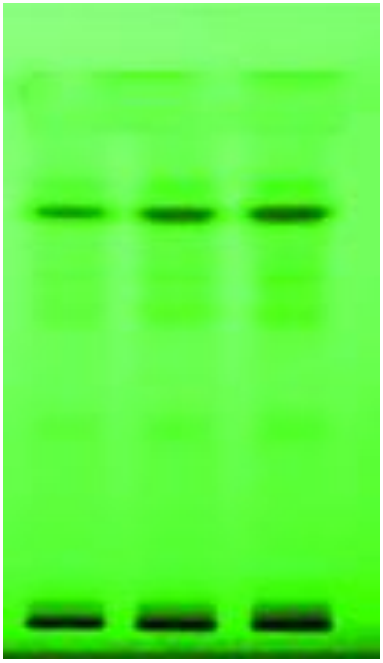
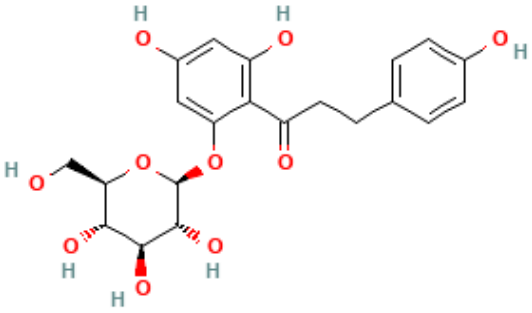


Figure 9: HPTLC of of Compound II



Figfure 10: Structure of Compound II (Phlorizin)

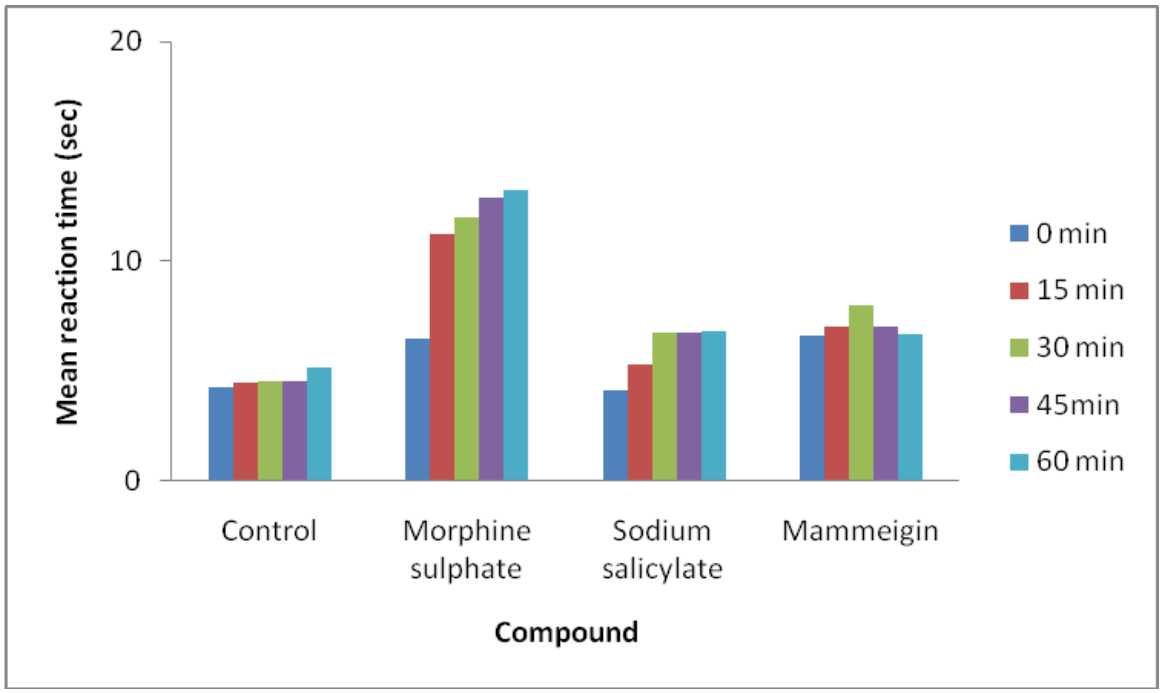


Figure 11: Analgesic effect of Mammeigin isolated compound by tail-flick method in rats

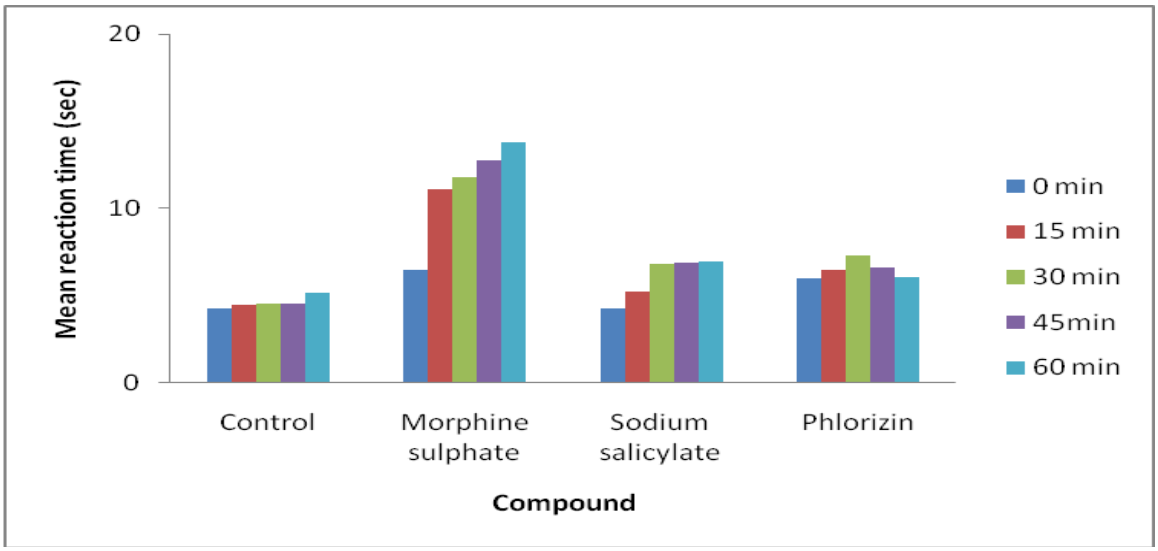


Figure 12: Analgesic effect of Phlorizin isolated compound by tail-flick method in rats

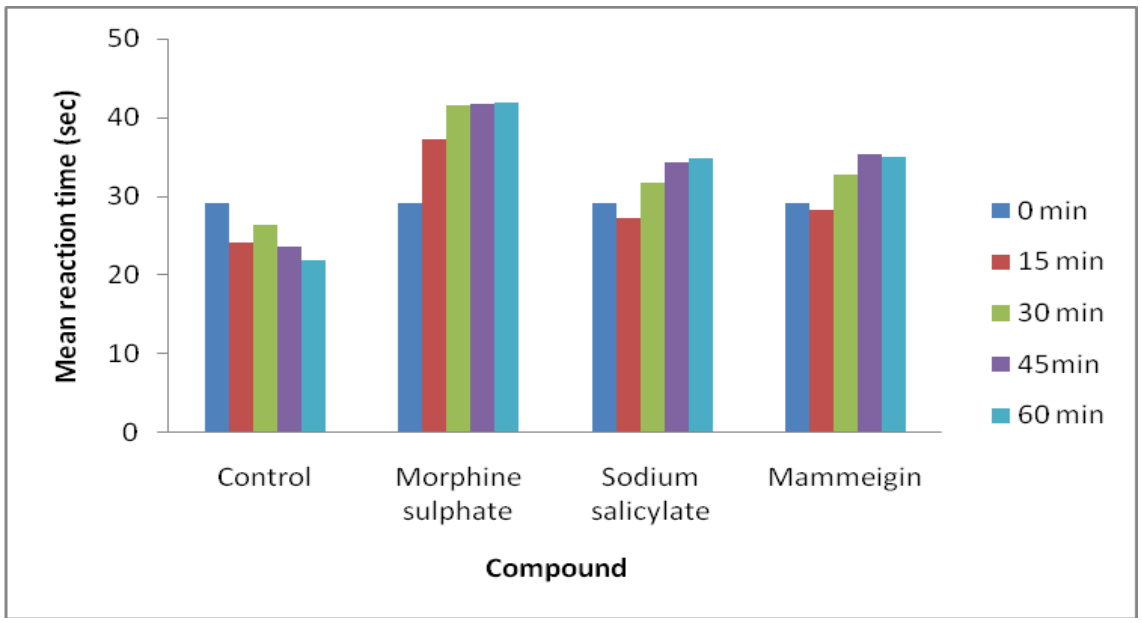


Figure 13: Effect of Mammeigin isolated compound by hot plate test method

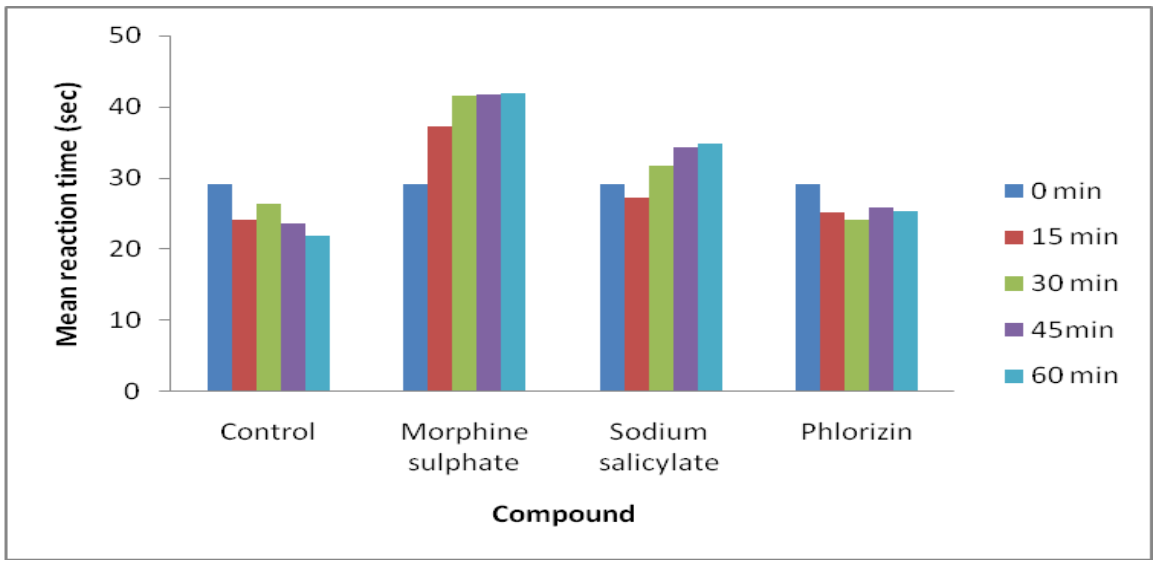


Figure 14: Effect of Phlorizin isolated compound by hot plate test method

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