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Book by two authors

Singh, U.B. and Sulochana, S. 1996. *Handbook of Histological and Histochemical Techniques.* Premier Publishing House, Hyderabad, 111p.

Book by more than two authors

Dyce, K.M., Sack, W.O. and Wensing, C.J.G. 1996. *Textbook of Veterinary Anatomy*.(2nd Ed.). W.B. Saunders Company, Philadelphia, 856p.

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KVASU [Kerala Veterinary and AnimalSciencesUniversity]. 2012. *Academic Handbook.* (1stEd.).Kerala Veterinary and AnimalSciencesUniversity, Pookode, 172p.

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Journal article by one author

Rao, G.S. 1991. Ovine hippocampus.*Indian J. Anim. Sci.***61**: 168-169.

Journal article by two authors

Gupta, S.K. and Sharma, D.N. 1990. Biometry of the bovine skull.*Indian J. Anim. Res.***24**: 110-114.

Journal article by multiple authors

Pramod, S., Nair, N.D., Ambily, V.R., Hiron, M., Vijayan, N. and Nair, G.K. 2012.Pathology of lymphoid organs in experimental duck cholera.*Indian Vet. J.* **89**: 20-22.

Journal article accepted for publication (but not yet published)

Malik, K.C., Mishra, P.C., Mishra, P.K. and Panda, N. 2013. Study on seasonal variations in seminal characteristics of synthetic colour cocks. *Indian J. Vet.Anat.* (in press).

Thesis

Arunima, S.S. 2011. Postnatal development of lymph nodes in Kuttanad duck (*Anasplatyrhynchosdomesticus).M.V.Sc thesis*, Kerala Veterinary and AnimalSciencesUniversity, Pookode, 66p.

Proceedings

Jayant, G., Peethambaran, P.A., Jalaludeen, A., Narayanankutty, K and Ally, K. Utilisation of dried cuttle fish (*Sepia officialis*) waste silage in layer duck ration. In: Jalaludeen, A. (ed.), *Proceedings of IV World Waterfowl Conference*; 11th to13th November, 2009, Mannuthy. Kerala Agricultural University, Centre for Advanced Studies in Poultry Science, College of Veterinary and Animal Sciences and World's Poultry Science Association (India branch). pp. 198-200.

Conference abstract

Shiju, S., Ramani, C., Rao, G.D. and Kannan, T.A. 2012. Traumatic ocular proptosis in Pug and its management [abstract]. In: *Compendium, National Symposium on Advances in Applied Anatomy of Domestic and Wild Animals- an Interdisciplinary Approach for Animal Health and Wealth*; 28th to 30th November, 2012, Mannuthy. Kerala Veterinary and AnimalSciencesUniversity.p. 17.Abstract No. 3.7.

Technical bulletin/series/reports

Evans, D.O. and Rotar, P.P. 1987. *Sesbania in Agriculture.*Westview Tropical Agriculture Series No. 8, Westview Press/ Boulder, London. l92p.

Patent

Smith, P. L. 2002. Particle trap for compressed gas insulated transmission systems, US Patent No. *4554399.*

Article from a weekly

Dutta, S. 2013, Feb. 13. Getting closer through culture.*Frontline*.**30**(2):94-96.

Article from a newspaper (with author)

Gargi, P. 2013, Jan. 16. Subsistence farmers must raise themselves. *The Hindu.*p.13.

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Book available only on the internet

Jarrard, R.D. 2001.*Scientific Methods* [book on-line]. Dept. of Geology and Geophysics, University of Utah.Available:http://www.mines.utah.edu/geo/people/faculty/jarrard/Text/booktoc.html. [30 Oct. 2003].

Printed journal article freely available on the internet

Chowdhury, S. and Sharma, B. 2013. Transcription of gD and gI genes in BHV1-infected cells. *J. Biosci.* **37**(6): 971-977. Available: http://www.ias.ac.in/jbiosci/dec2012/971.pdf. [22 Jan. 2013].

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Kumar, P., Kumar, S. and Singh, Y. 1995a.Anatomy of the pineal gland in domestic animals.*Indian J. Vet. Anat.***7**: 1-10.

Kumar, P., Kumar, S. and Singh, Y. 1995b.Topography and histomorphology of pineal gland in young goat.*Indian J. Anim. Sci.***65**: 633-635.

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JVAS- Model Research Article

Qualitative and quantitative analysis of methanol extract of *Crataevanurvala* stem bark[#]

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Abstract

Medicinal plants are precious source of bioactive compounds which possess a range of beneficial properties and they remain the major source of medicine for a large proportion of population in this world. From ancient time, Crataevanurvala was used as a vital herb in Ayurvedic system of medicine. According to Unani system of medicine, bark of C. nurvala is used as an appetite stimulant and as an agent to decrease the secretion of bile and phlegm. In the present study, methanol extract of stem bark of C. nurvala was analysed for preliminary phytochemicals and chemical profiling of the extract was illustrated using gas chromatography and mass spectrometry (GC-MS) analysis. The phytochemical analysis revealed that the plant extract contained alkaloids, steroids and triterpenoids. Gas chromatography mass spectrometry analysis determined the presence of different compounds of biological importance. The identification and characterisation of the phytoconstituents in the extract could pave the way for the discovery of new drugs for various ailments.

[#]*Part of MVSc thesis submitted to Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala*

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Keywords: *Crataevanurvala, triterpenes, alkaloids, steroids*

Running title: Qualitative and quantitative analysis of *Crataevanurvala* stem bark

Medicinal plants are valuable source of naturally active phytochemicals. They are the naturally occurring chemical compounds found in plants which provide health benefits for humans and animals. These compounds commonly known as secondary plant metabolites have been attributed to have different biological properties providing protection against various diseases.

*Crataevanurvala*Buch-Ham., commonly known as Varuna, Neermathalam, BarnaChal, belonging to the family of Capparidacea, is a moderate sized deciduous tree. A variety of medicinal properties have been reported for *C. nurvala* and its stem bark. It has been traditionally used in treating blood flow, waste elimination, breathing problems, fever, metabolic disorders, joint lubrication and wound healing (Vashist*et al*., 2020). Mekap*et al.* (2011) determined the antiurolithiatic activity of *C. nurvala*. Root and bark are documented to be laxative, lithotripsic and was found to increase the appetite and biliary secretion (Fletcher, 1993; Malini*et al.*, 1995). The ethanol and aqueous extracts of the dried stem bark of *C. nurvala* have been found to possess significant anti-fertility effects in rats (Bhaskar*et al*., 2009). The antidiarrhoeal activity of ethanol extracts of *C. nurvala* stem bark has been reported by Inayathulla*et al*. (2010). *Crataevanurvala* stem bark extract exhibited antidiabetic activity against alloxan induced diabetic albino rats in the study done by Sikarwar and Patil (2010). Thus, the present study was carried out to evaluate the various phytochemical constituents present in the bark of methanol extract of *C. nurvala* which would be helpful to delineate the various biological activities shown by the stem bark.

Materials and methods

Plant collection and identification

The bark of *C. nurvala* was collected from Valluvanad, Palakkad, Kerala (Fig. 1 and 2). The collected plant material was identified and its authenticity was confirmed by Raw Material Herbarium and Museum (RHMD), NISCAIR, New Delhi, India.

Fig. 1. Leaves and flower of *Crataevanurvala*Buch-Ham.

Fig. 2. Bark of *Crataevanurvala*Buch-Ham.

Preparation of extracts

Freshly collected bark of *C. nurvala*were cleaned to remove adhering dust and then dried under shade. The dried bark was coarsely powdered using an electric pulveriser and the powder obtained was extracted using a Soxhlet apparatus with methanol at 67 ^{o}C. The methanol extract was then concentrated using a rotary vacuum evaporator under reduced pressure and temperature (40 ^{o}C). The yield of the extract was calculated using the formula: Yield value (%) = Extracts obtained/ Total amount of crude drug × 100, and kept under refrigeration in an airtight container after complete evaporation of the solvent for further use.

Qualitative phytochemical analysis

The extracts were tested for the presence of bioactive compounds using methods described by Harborne (1998). Fifty milligrams of the extract were dissolved in 3 mL of chloroform. Few drops of concentrated sulphuric acid were added and the solution was allowed to stand. Formation of red colour directed the presence of steroids.

GC-MS analysis

The active phytochemical principles of methanol extract of *C. nurvala*was analysed using GC-MS system of Centre for Analytical Instrumentation- Kerala(CAI-K), Kerala Forest Research Institute (KFRI), Peechi, Kerala. The GC-MS analysis was carried out on Gas chromatography Mass Spectrometer (Shimadzu GC-MS, Japan, QP2010SE) with a mass range of 1.5- 1000 m/z. Helium at a flow rate of 1 mL/ min was used as the carrier gas. The oven temperature was maintained at 80 °C for 4 min and then increased to 280 °C in 6 minutes. The injector temperature was260 °C andtotalanalysistimewas50minutes.Aliquotof the extract (0.4 µL) was injected into the chromatographic column after obtaining a clear baseline. The interpretation of the mass spectrum of GC-MS was guided using the database of the National Institute of Standards and Technology (NIST 11) and WILEY 8. The spectrum of the unknown compounds was related with the spectrum of the known compounds. The name and molecular weight of the compounds of the tested materials were ascertained.

Results and discussion

Qualitative phytochemical analysis

The qualitative phytochemical screening of methanol extract of stem bark of *C. nurvala* showed the presence of steroids, triterpenoids and alkaloids (Arunima, 2011). Phytochemical screening of methanol extract of stem bark of *C. nurvala* revealed the presence of steroid and terpenoids as well as alkaloids, phenolics, flavanoids, tannins and saponins (Hade *et al.*, 2016) which supported our results. Sodipo*et al.* (2000) have reported that alkaloids have been

associated with medicinal uses for centuries and one of their common biological properties is their cytotoxicity. Huang*et al.* (2016) isolated six phytosteroids and nine known triterpenoids from the leaves of *Chisochetoncumingianus* in which chisopanoidsE and F exhibited potent cytotoxicities towards MCF-7 with IC_{50} values of 3.24 ± 1.39 and 8.85 ± 4.73 μM, and were further proved to prevent the cell proliferation, mainly by inducing apoptosis.Haque*et al.* (2008) isolated two terpenoids, phragmalin triacetate and lupeol from ethyl acetate extract of stem bark of *C. nurvula* by chromatographic techniques. Jain *et al.* (2016) suggested that terpenoids were capable of inhibiting NFκB through different mechanisms. Khatun*et al.* (2015) evaluated the antioxidant, anthelmintic, antimicrobial and phytochemical assessment of ethanolic extract of *C. nurvala* leaves and displayed the presence of alkaloids, flavonoids, reducing sugar, saponins, steroids, tannins. The above mentioned phyto constituents are described to exhibit various pharmacological activities (Table 1).

Table 1. Results of analysis of calculi in Fourier Transform Infrared Spectrometry with Attenuated Total Reflectance (FTIR-ATR)

Functional Group Assignments	**Animal no.**	**Reported IR wavelength (cm^{-1})**	**Standard IR wavelength of pure struvite (cm^{-1})**
H-O-H stretching vibrations of water of crystallization	A_1	3401.94	3270
	A_2	3391.81	
	A_3	3500-3350	
	A_4	3360.29	
H-O-H stretching vibrations of a cluster of water molecules	A_1	-	2385
	A_2	2331.52	
	A_3	2346.67	
	A_4	2321.69	
H-O-H bending modes of vibrations	A_1	1440.19	1445
	A_2	1434.90	
	A_3	1434.68	
	A_4	1441.17	
N-H symmetric stretching vibrations in NH4+ units		-	2935
N-H symmetric	A_1	3401.94	3270

stretching vibrations	A_2	3391.81	
	A_3	3500-3350	
	A_4	3360.29	
N-H symmetric stretching vibrations in NH4+ units		-	2935
N-H bending vibration	A_1	1670.28	1666
	A_2	1650.70	
	A_3	1651.8	
	A_4	1654.67	
N-H asymmetric bending vibration in NH4+ units Ionic phosphate	A_1	995.16	1010
	A_2	1000.4	
	A_3	1000.48	
	A_4	1000.14	

Standard values used as per Bindhu*et al.*, 2012

GC- MS analysis

The results of GC-MS analysis of methanol extract revealed the presence of twenty-one compounds. The GC-MS chromatogram of twenty-one compounds is depicted in Fig. 3. Thymine, 3-hydroxy-2,3-dihydromaltol, 5-hydroxymethylfurfural, n-methyl-3-hydroxymethyl pyrrolidine-2-one, cytidine, methyl pentofuranoside, undecane, 6,6-dideutero-5-methyl-, 2,4-ditert-butylphenol and 3-deoxy-d-mannoic lactone were the major compounds.

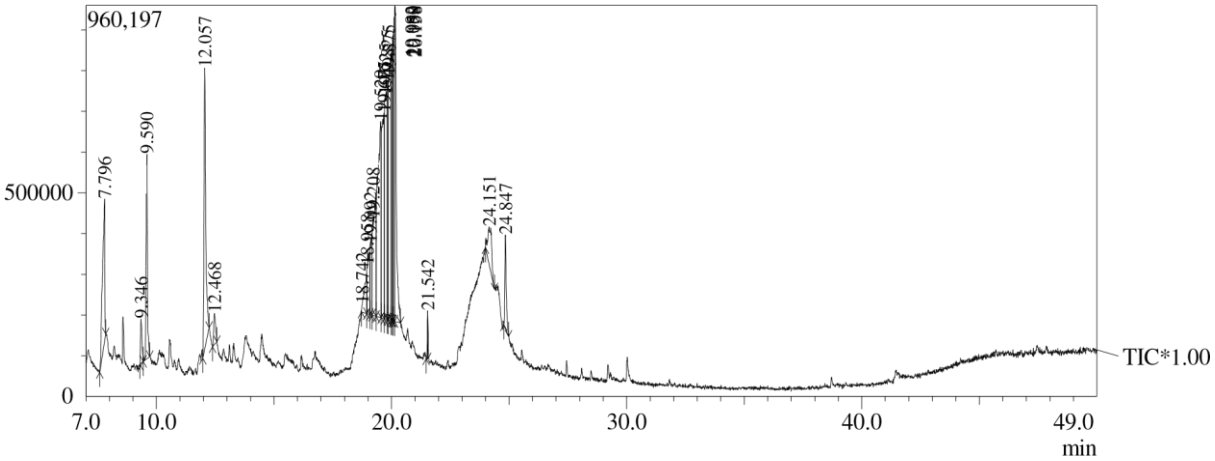

Fig. 3. GC-MS chromatogram of methanol extract of *C. nurvala*stem bark

Balamurugan *et al.* (2019) havedone the chemical profiling ofmethanolbark extract of *C. nurvala*using GC-MS technique. The study revealed the presence of 8 components such as lup-20 (29)-en-3ol, 2-hydroxy-4methoxybenzaldehyde, methoprene, 1'-acetonaphthone, 1, 2-bis (Trimethylsilyl) benzene, pivalate, cyclotrisiloxane, limonen-6-ol and 4-hexadecen-6-yne .

The recognised major compounds in our study possess some significant biological activities for future drug development.Zhao *et al.* (2013) showed that 5-hydroxymethylfurfural (5-HMF) induced apoptosis and G0/G1 cell cycle arrest in human melanoma A375 cells. Takuli*et al.* (2020) elucidated the antioxidant and antibacterial activity of *Woodwardiaunigemmata* (Makino) along with chemical characterisation which revealed the presence of 3-hydroxy-2,3-dihydromaltol in GC-MS analysis. Azizi*et al.* (2006) performed fast gas chromatography/ time of flight mass spectrometry (TOF-GCMS) which identified N-methyl-3-hydroxymethylpyrrolidin-2-one from the oil extract of *Pithecellobiumjiringan* jack seeds which was found to abolish excess free radicals and counteract oxidative damage. Su*et al*. (2005) evaluated the antioxidant activity of methanol extract of *Morindacitrifolia* (Noni) fruits and the purification of its butanol soluble partition of methanol extract contained isolates like cytidine. Shaheed*et al*. (2018) identified methyl pentofuranoside, also known as alpha-d-mannofuranoside, from methanolic fenugreek seed extract and determined its antibacterial activity against *Streptococcus agalactiae, Escherichia coli, Enterococcus cloacae* and *Proteus mirabillis.* Gas chromatography mass spectroscopic analysis exhibited the presence of undecane,6,6-dideutero-5-methyl- in *Nigella sativa*, *Allium sativum*, *Propolis* and *Oleaeuropaea* mixture which was depicted as antibacterial and antifungal agent (Bintang*et al.*, 2018). Chuah*et al*. (2015) suggested that 2,4-di-tert-butylphenolinduced oxidative stress through the generation of reactive oxygen species, which cause lipid peroxidation and membrane damage in root tissues and chloroplast in leaf tissues, thus leading to increased levels of antioxidant enzymes. Shobana *et al.* (2009)in their study identified compounds such as 3-deoxy-d-mannoic lactone and thymine from two varieties of garlic (ophioscordon and sativum) which was found to possess antibacterial activity

against enteric pathogens. The aforesaid isolated compounds from the methanol extract of *C. nurvala* stem bark seemed to own the reported biological activity and further study of these phytoconstituents may demonstrate the medicinal importance in future. The biological activities of other compounds have not been reported so far and more study of these phytoconstituents might validate the significant medicinal features in forthcoming.

Conclusion

The association among the phytochemical constituents with their biological activities is now being the matter of advanced thought. *Crataevanurvala* is a deciduous medium sized tree, traditionally used in the treatment of kidney stones, urinary tract infection and prostate related disorder. The present study has revealed *C. nurvala* to be rich in various phytochemicals. The existence of these phytochemical constituents indicated that the bark of the plant could be used in a variety of ways which would be beneficial to the population. Gas chromatography mass spectroscopic analysis revealed the presence many compounds presumed to be responsible for eliciting the traditional medicinal activities of the bark of the plant.

Acknowledgement (If any)

Conflict of interest

The authors declare that they have no conflict of interest.

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JVAS- Model Short Communication

Copro-polymerase chain reaction for molecular identification of *Haemonchuscontortus*in goats

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Abstract

Haemonchuscontortus commonly called as stomach worm or wire worm of ruminants inhabit the abomasum and is considered as one of the economically important gastrointestinal strongyles in goats. In the present study, H. contortus was identified by PCR using the primers targeting partial 5.8S and partial internal transcribed spacer region 2 (ITS-2). Adult worms were identified morphologically and genomic DNA was extracted using DNeasy Blood and Tissue kit (QIAGEN, Germany). Gradient PCR protocol was standardised using the extracted genomic DNA. Ten-fold serial dilution of adult DNA was used to analyse the minimum detection limit and the products were amplified upto tenth dilution. Cross reaction of primer sets was checked using the DNA extracted from predominant adult strongyles like Oesophagostomumcolumbianum and Trichostrongyluscolubriformis and no cross reaction was seen at the optimum annealing temperature (60.7°C)

Keywords: *Haemonchuscontortus, goats, PCR, ITS-2*

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Short Running Title:Molecular identification of *Haemonchuscontortus*in goats

Haemonchuscontortus belong to Trichostrongylidae family and are commonly called as stomach worm or wire worm of ruminants. The adult worms attach to the abomasal mucosa of small ruminants and due to its haematophagus nature it causes anaemia, jowl oedema and even death in young ones. Adult worms are identified based on morphological features. But identification of nematode species based on features of strongyle egg is difficult during coprological examination. Coproculture aided in species identification but it takes seven to ten days to identify the infective larvae (Fletcher, 1993). Hence, molecular identification was undertaken in this study as a tool for species level identification.

Table 1. Composition of reaction mix for PCR to identify infective larvae of *H. contortus*

Components	Quantity (µL)
10 X PCR buffer (without $MgCl_2$)	2.5
dNTP (10 mM each)	0.50 (200µM each)
Primer forward	1(10 pmol)
Primer reverse	1 (10 pmol)
Magnesium chloride (25 mM)	1.50 (1.5mM)
*Taq*polymerase (5 IU/µL)	0.20 (1U)
DNA template	5
Nuclease free water	13.3
Total	25

After performing gradient PCR, the amplicons were subjected to agarose gel electrophoresis in 1.5 per cent agarose gelat 80V, 400mA for 35 min and the gel was visualised in Gel Doc^{TM}EZ imager and documented using Image lab software.The amplicons were purified and sequenced at AgriGenom labs private limited, Cochin using Sanger's di-deoxy nucleotide chain termination method.

Male tail end had well developed bursa with elongate lateral lobes which was supported by an asymmetrical dorsal lobe. Dorsal lobe was placed on the left lateral lobe which was supported by an inverted Y shaped dorsal ray. Whereas, female had barber's poleappearance which was due the coiling of white ovaries wound around the red intestine(Soulsby, 1982).*Haemonchuscontortus*has been identified as the predominant strongyle species in goats in different places including Kerala (Deepa, 2005), North-West India (Kumar *et al*., 2008) Malaysia (Chandrawathani*et al*., 2009), Kashmir (Irfan-ur-Rauf-Tak*et al*., 2013) and Ethiopia (Chalchisa*et al*., 2015).

Sensitivity of *H. contortus* primer sets was checked using ten-fold serial dilution and the ability of primers to amplify minimum DNA concentration was analysed. The initial concentration of DNA used for sensitivity study was 4.7 ng/µL and ten-fold serial dilution was performed. PCR products were amplified upto tenth dilution which showed that the primer pairs could amplify DNA with minimum concentration of 4.7 ag/ µL (attogram per microlitre) (Fig. 1).

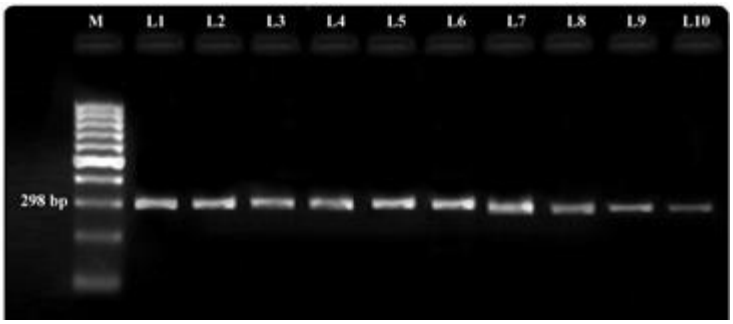

Fig. 1. Ampliconsof *H. contortus*
Lane M: 100 bp ladder
Lane 1-10: Ten-fold serial dilution *H. contortus*DNA

The specificity of primer was cross checked with DNA of other important strongyles like *Oesophagostomumcolumbianum*and*Trichostrongyluscolubriformis* to detect the cross amplification between species.

Summary

The study forms the basis for developing copro-polymerase chain reaction for detecting *H. contortus* infection in goats. Specific detection of this pathogen from clinical samples would aid in initiating timely control measures.

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Conflict of interest

The authors declare that they have no conflict of interest.

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